Measurement of Hepcidin-20, -22, -24, and-25 in Human Serum by Liquid Chromatography-High Resolution-Mass Spectrometry and its Clinical application

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Measurement of Hepcidin-20, -22, -24, and-25 in Human Serum by Liquid Chromatography-High Resolution-Mass Spectrometry and its Clinical application

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Abstract

Hepcidin-25 is regarded as the master regulator of iron homeostasis. Three *N*-truncated isoforms of hepcidin-25 have been identified in human serum; hepcidin-20, -22, and -24, although information is scant as to the serum concentrations of these isoforms.

A liquid chromatography-high resolution-mass spectrometry (LC-HR-MS) assay was developed for the simultaneous quantitation of hepcidin isoforms in human serum. Serum (200 µL) was mixed with aqueous formic acid (600 µL), and the supernatant loaded onto a 96-well-SPE-plate. Eluted sample (70 µL) was diluted with deionised water (60 µL) and analysed using LC–HR–MS. Samples previously analysed by a published LC-MS/MS assay were analysed for method comparison. All hepcidin isoforms were quantified in samples from healthy volunteers as controls, and patients with hereditary haemochromatosis (HH), non-alcoholic fatty liver disease (NAFLD), iron deficient anaemia (IDA), anaemia of chronic disease (ACD), and sickle cell anaemia (SCA). Samples were also analysed from individuals with chronic kidney disease (CKD) not requiring haemodialysis, and those pre- and post-haemodialysis.

Intra-/inter-assay accuracy and precision were acceptable, calibration was linear (R^2 > 0.90, all analytes), and the LLoQ was 1 µg/L (all analytes). There was a good correlation for hepcidin-25 to a published LC-MS/MS assay (y = 0.85x -3.2, R^2 = 0.96). Median (range) hepcidin-25 concentrations in controls, and individuals with IDA, SCA, HH, ACD and sepsis were: 8 (1–31), <1 (<1–2), <1 (<1–10), 2 (<1–15), 60 (10–213), and 92 (11–216) µg/L, respectively. Hepcidin-20, -22, or -24 were not detected in any control sample, but were detected in 30–100 % of all samples at 10–20 % of the hepcidin-25 concentration. Following haemodialysis, all hepcidin isoforms declined by some 35–50 %. Hepcidin-25 was most strongly correlated to hepcidin-24, and less so to hepcidin-22 and -20, in all disease states.

The developed method was applicable for clinical use. However, further controlled studies are required to fully evaluate the role of hepcidin-20, -22, and -24 measurement in a clinical setting.

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Abbreviations

Abbreviation	Term
3-NBA	3-Nitrobenzyl Alcohol
a.p.s	Average Particle Size
ACD	Anaemia of Chronic Disease
BD	Beckton Dickson
BMP	Bone Morphogenetic Protein
CDA	Congenital dyserythropoietic anaemia
CKD	Chronic Kidney Disease
cRIA	Competitive Radioimmunoassay
CRP	C-Reactive Protein
D.A.R.T	Disposal Automated Research Tips
DIOS	Dysmetabolic iron overload syndrome
DMT-1	Divalent metal-ion transporter-1
DCYTB	Duodenal cytochrome B
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
ESA(s)	Erythropoietin Stimulating Agent(s)
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FP-1	Ferroportin-1
GDF15	Growth/differentiation factor 15
HAMP	Hepcidin antimicrobial peptide
HB	Haem bound
HbC	Haemoglobin C
HbS	Haemoglobin S
HCP-1	Haem carrier protein 1
HFE	Human Factors Engineering
HJV	Haemojuvelin
HLB	Hydrophilic lipophilic balanced
HO-1	Haemoxygenase 1
HPLC	High Performance Liquid Chromatography
HR-MS	High Resolution-Mass Spectrometry
HRG1	Heme-responsive gene 1 protein homolog
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
ID	Iron-Deficiency
IDA	Iron Deficient Anaemia
IL-6	Interleukin-6
IQC	Internal Quality Control
IV	Intra-Venous
KCH	King's College Hospital
LC	Liquid Chromatography
LC-HR-MS	Liquid Chromatography-High Resolution-Mass Spectrometry

Abbreviation	Term
LC-MS(MS)	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LEAP	Liver expressed antimicrobial peptide
LLoQ	Lower Limit of Quantitation
LR-MS	Low Resolution-Mass Spectrometry
m/z	Mass-to-charge ratio
MALDI	Matrix Assisted Laser Desorption Ionisation
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionisation-Time Of Flight-Mass spectrometry
MAX	Maximum anion exchange
MCX	Maximum cation exchange
ME	Matrix Effect
MS	Mass spectrometry
MSIA	Mass Spectrometric Immunoassay
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHB	Non-haem bound
NRAMP-1	Natural Resistance-Associated Macrophage Protein-1
P20	Polysorbate20
PE	Process Efficiency
pI	Isoelectric point
ppm	Parts per million
PPT	Protein Precipitation
RE	Extraction Recovery
SCA	Sickle Cell Anaemia
SDS	Sodium Dodecyl Sulfate
SELDI-MS	Surface Enhanced Laser Desorption Ionisation-Mass Spectrometry
SELDI-TOF-MS	Surface Enhanced Laser Desorption Ionisation-Time Of Flight-Mass Spectrometry
SLC40A1	Solute Carrier family 40 (iron-regulated transporter), member 1
s-HJV	Soluble Haemojuvelin
SHS	Stripped Human Serum
SMAD	Sma Mothers Against Decapentaplegic
SPE	Solid Phase Extraction
SRM	Selective-Reaction-Monitoring
STEAP	Six Transmembrane Epithelial Antigen of the Prostate
STfR	Soluble Transferrin Receptor-1
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TfRI, TfRII	Transferrin Receptor-I/II
TIBC	Total Iron Binding Capacity
TOF-MS	Time Of Flight-Mass Spectrometer/y
TSAT	Transferrin saturation (expressed as a percentage)

Abbreviation	Term
TWSG1	Twisted Gastrulation BMP Signalling Modulator 1
UV	Ultra-Violet
v/v	Volume to volume ratio
WAX	Weak anion exchange
WCX	Weak cation exchange

Chapter 1 Introduction

1.1 Introduction

Iron is an essential element that is required for adequate erythropoietic function, oxidative metabolism, and cellular immune response. Even though iron is a vital element, free or 'non-protein bound' iron can be extremely toxic, causing damage to cellular membranes, proteins, and DNA through the formation of reactive oxygen species via the Fenton reaction (von Drygalski & Adamson, 2013). Therefore, iron uptake, transport and storage are highly regulated. The regulation of iron metabolism is mediated by several complex pathways, and although the fundamental principles are well understood, the exact mechanisms of all pathways remain unsolved. In 2000, hepcidin-25, a peptide hormone, was identified in human serum (Krause *et al.,* 2000) and urine (Park *et al.,* 2001), and is now considered to be the master regulator of iron homeostasis. The identification and study of hepcidin-25 has greatly improved our understanding of the physiology and pathophysiology of iron homeostasis, in-part initially through the use of immunochemical based methods. A number of promising areas have been suggested for the measurement of hepcidin-25, these include screening for hereditary haemochromatosis (HH) and the treatment of iron deficiency or overload with hepcidin-25 antagonists or agonists. The development of accurate, precise, and selective analytical methods for the measurement of hepcidin-25, however has been challenging (Kroot *et al*., 2009; Kroot *et al*., 2012; van der Vorm *et al*., 2016) but is crucial if hepcidin-25 measurement is to be used routinely in clinical diagnosis and management of patients.

1.2 Iron

1.2.1 Absorption

Non-haem bound (NHB) iron is absorbed from the proximal duodenum. In this acidic environment, NHB iron is maintained in the ferric (Fe^{3+}) state, but is reduced to ferrous iron (Fe^{2+}) by the ferrireductase duodenal cytochrome B (DCYTB, Figure 1-1, McKie *et al.,* 2001), where it is transported into the enterocyte via the divalent metal-ion transporter-1 (DMT-1) (Gunshin *et al.,* 1997). Haem bound (HB) iron is transported across the apical membrane via haem carrier protein-1 (HCP-1) (Shayeghi *et al.,* 2005). Once in the enterocyte NHB iron is either bound to ferritin - the primary iron storage protein that can store up to 4000 atoms of iron per molecule, or exported from the cell via ferroportin-1 (FP-1) (Donovan *et al.,* 2000; McKie *et*

al., 2000). Once HB iron is within the cell, it is processed by haemoxygenase-1 (HO-1) to liberate Fe^{2+}. This is then either bound to ferritin or transported across the basolateral membrane by FP-1. Iron released through FP-1 is re-oxidised to the ferric form by the membrane-bound ferroxidase; hephaestin (Fuqua *et al.,* 2014; Vulpe *et al.,* 1999). Once in the plasma, iron is transported by transferrin, a single chain polypeptide produced in the liver that can bind two molecules of ferric iron.

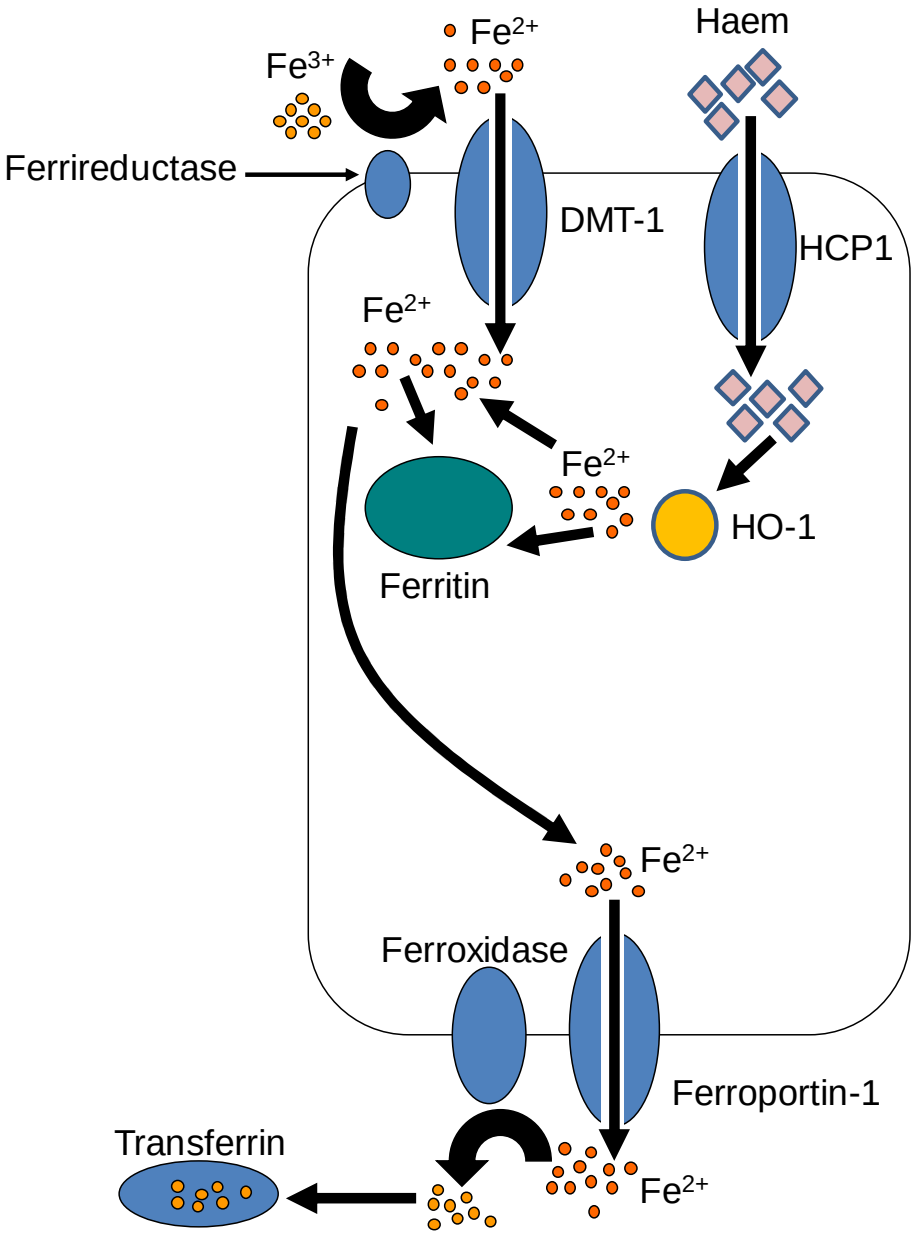


Figure 1-1. Schematic showing absorption of haem and non-haem bound iron by enterocytes. (DMT-1: divalent metal-ion transporter-1; HCP-1: haem carrier protein 1; HO-1; Haemoxygenase 1).

1.2.2 Transport and Erythropoiesis

Once in circulation, most iron bound transferrin is transported to the bone marrow for use in developing erythroid cells. At the cell membrane, the iron-transferrin complex binds to Transferrin Receptor-1 (TfR1), and enters the cell via clathrin-coated pits to form clathrin-coated endosomes (siderosomes). Within the cell, following the removal of clathrin, the siderosomes are acidified by an ATP-dependent proton pump (V-type ATPase). This change in pH alters the structure of transferrin and TfR1, releasing Fe^{3+}. Ferric iron is then reduced to Fe^{2+} by the ferrireductase STEAP (six transmembrane epithelial antigen of the prostate) proteins and transported to the cytoplasm via DMT-1 (Dautry-Varsat *et al.,* 1983, Ohgami *et al.,* 2005, 2006). Once in the cytoplasm, the released iron is transported to mitochondria, where it is incorporated into haemoglobin. Once the erythrocyte is fully mature and ready for release into the circulation, the transferrin protein is released back into circulation, and TfR1 is released back to the cell membrane. At the cell membrane TfR1 is cleaved by proteases and is released into the plasma as soluble TFR1 protein (STfR) (Hentze *et al.,* 2010). Therefore, the concentration of STfR in the plasma reflects the iron status of the patient, and total erythropoetic activity.

1.2.3 Recycling and Storage

Macrophages play an important role in iron recycling, as senescent erythrocytes are phagocytosed by macrophages present in the reticuloendothelial system. While in the macrophage, ferrous iron is released into the cytosol by HRG1 (Heme-responsive gene 1 protein homolog) where haem catabolism takes place (White *et al*., 2013). Iron is then exported from macrophages to transferrin by FP-1 (Hentze *et al.,* 2010). Macrophages are also an important store for iron. In the cell, iron can either be stored as ferritin in the cytosol, or as haemosiderin. When iron is stored within macrophages it is relatively inert, that is, reactive oxygen species are not formed and oxidative damage does not occur. Usually, storage of iron as haemosiderin within macrophages is minimal, but can increase dramatically during iron overload. The liver is also another main storage organ for iron, and during iron overload, reactive oxygen species are generated leading to lipid peroxidation, cell necrosis, and progressive liver injury.

1.3 Hepcidin

1.3.1 Synthesis and structure

Hepcidin-25, is a 25 amino acid polypeptide that is primarily synthesised within the liver as an initial 84 amino acid 'prepropeptide' that is encoded by the hepcidin antimicrobial peptide gene (*HAMP*), also known as liver expressed antimicrobial peptide (*LEAP*). The first 24 amino acids contain an endoplasmic reticulum targeting signal, and cleavage of the 84 amino acid prepropeptide produces a 60 amino acid long 'prohepcidin'. Prohepcidin is then further cleaved by prohormone convertase furin to produce hepcidin-25 (Figure 1-2; Macdougall *et al.,* 2010; Valore & Ganz., 2008).

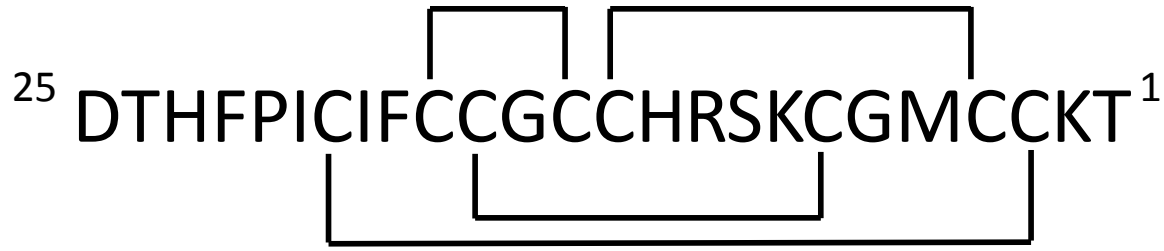


Figure 1-2. Amino acid sequence of hepcidin-25, with cysteine bonds indicated (1 = *C*-terminus, 25 = *N*-terminus).

Loss of amino acids from the *N*-terminus of hepcidin-25 can result in the formation of hepcidin-20 (20 amino acids), hepcidin-22 (22 amino acids) and hepcidin-24 (24 amino acids), all of which have been identified in human urine, plasma or serum (Addo *et al.,* 2016; Campostrini *et al.,* 2012; Laarakkers *et al.,* 2013; Moe *et al.,* 2013; Rochat *et al.,* 2013). Other *N*-truncated isoforms; hepcidin-19 (19 amino acids), hepcidin-21 (21 amino acids), and hepcidin-23 (23 amino acids) have also been reported in human serum (Moe *et al.,* 2013). This finding, however, has not been substantiated by other investigators, and it remains to be ascertained whether these were an artefact of sample storage and/or preparation prior to analysis.

Little is known as to the origin of the smaller *N*-truncated hepcidin isoforms. It has been suggested that calcium-independent tissue activity in pancreas extracts may lead to the *N*-terminal processing of hepcidin-25 to hepcidin-22, and that dipeptidylpeptidase-4 maybe involved in the formation of hepcidin-20 from hepcidin-22 (Schranz *et al.,* 2009, Valore and

Ganz, 2008). However, evidence is scarce and these isoforms of hepcidin may be *ex-vivo* artefacts of hepcidin-25 degradation during storage or sample preparation.

All hepcidins have a distorted beta-sheet with a hairpin loop that is stabilised by four disulphide bridges and hydrogen bonds (Figure 1-3). All hepcidins are weakly basic with an equal number of hydrophobic, hydrophilic and charged amino acids within their sequences (Table 1.1), giving rise to an amphipathic structure containing both hydrophilic and hydrophobic regions, with a tendency to adhere to laboratory glass/plastic ware, and for the peptide to aggregate (Malyszko, 2009).

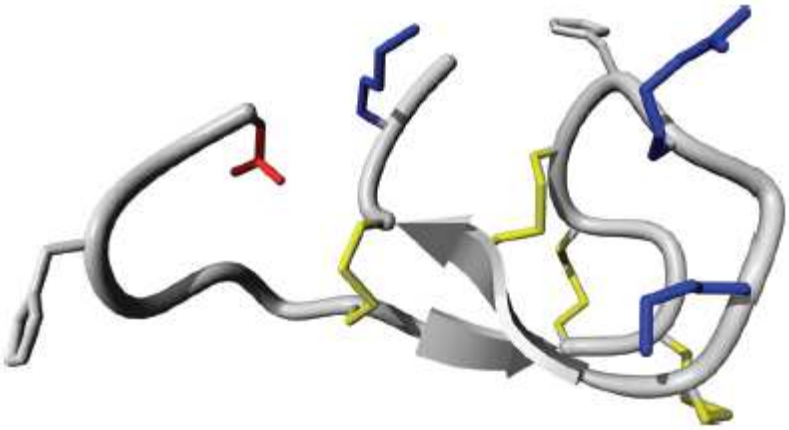


Figure 1-3. Molecular structure of hepcidin-25 [Distorted beta sheets (grey arrows), peptide sequence (grey), disulphide bonds (yellow), charged residues: positive (blue) and negative (red) (Jordan *et al.,* 2009)].

Table 1.1. Some physiochemical properties of hepcidin isoforms (isoelectric point data from http://web.expasy.org/compute_pi/, accessed 27/10/2016).

	Isoelectric point (pI)	**Hydrophobic amino acids (% of sequence)**	**Hydrophilic amino acids (% of sequence)**	**Charged amino acids (% of sequence)**
Hepcidin-19	8.53	16	21	21
Hepcidin-20	8.53	25	25	25
Hepcidin-21	8.60	24	19	19
Hepcidin-22	8.53	27	18	18
Hepcidin-23	8.53	26	22	22
Hepcidin-24	8.51	25	25	21
Hepcidin-25	8.22	24	28	24

1.3.2 Function and Regulation

Hepcidin-25 binds to, and causes the internalisation and proteolysis of FP-1 on the membranes of macrophages, enterocytes and hepatocytes (Nemeth *et al.*, 2004). This causes increased intracellular iron stores, decreased dietary iron absorption, and hence decreased circulating iron concentrations (Kroot *et al.,* 2011; Rizvi & Schoen, 2011). *In-vitro* studies have shown that hepcidin-20, -22 and hepcidin-24 have little, if any activity at the FP-1 receptor, and therefore are unlikely to have a significant effect on iron metabolism (Laarakkers *et al.,* 2013; Nemeth *et al.,* 2006). However, hepcidin-20, as well as hepcidin-25 have been shown to have bactericidal properties, although hepcidin-20 has a lower activity than hepcidin-25. The exact antibacterial mechanism is currently unknown, but may involve binding of copper within bacteria (Ho *et al.,* 2013; Lombardi *et al.,* 2015; Maisetta *et al.,* 2010).

Several processes are involved in regulating plasma hepcidin-25 concentrations - separated into positive and negative regulators. Hepcidin-25 synthesis, and subsequent increases or decreases in plasma iron occur within a few hours of exposure to appropriate stimuli. Studies in mice have shown that following intraperitoneal injection of synthetic hepcidin-25, serum iron concentrations declined by some 3-fold (Rivera *et al*., 2005). Positive regulators that increase hepcidin-25 plasma concentrations are primarily infection, inflammation and an increase in iron stores. During inflammation, the inflammatory cytokine IL-6 (interleukin-6) induces hepcidin synthesis via the janus kinase/signal transducer and activator of transcription-3 pathway (Nemeth *et al.,* 2004a). It has been suggested that this increase in plasma hepcidin-25 in response to infection and inflammation may have evolved as a host response to infection, where low plasma iron could slow the growth of micro-organisms (Fung & Nemeth, 2013).

A principal mechanism by which hepcidin-25 expression is stimulated is through the BMP-6-HJV-SMAD signalling pathway (Figure 1-4). With increasing iron stores, bone morphogenetic proteins (BMPs) form complexes with type I and II BMP receptors within hepatocytes, resulting in phosphorylation of SMAD proteins (Figure 1-4; Babitt *et al.,* 2006; Babitt *et al.,* 2007). The phosphorylated SMAD protein forms a complex with SMAD4, activating transcription of the *HAMP* gene. Other iron sensors such as transferrin receptors I and II (TFRI and II) are also involved in regulating hepcidin-25 in response to raised plasma iron, and the haemochromatosis protein (HFE) has been suggested to act as a shuttle between transferrin receptors, although the precise mechanism by which these proteins act is poorly understood

(Poli *et al.,* 2014). Haemojuvelin (HJV) is a member of the repulsive guidance molecule family, encoded by the *HFE-2* gene and exists in two forms, a cell membrane bound form and a soluble form (S-HJV). Membrane bound HJV is a co-receptor for BMP receptors and acts with BMP-6 to stimulate hepcidin-25 expression via the SMAD pathway within hepatocytes (Babitt *et al.,* 2006; Casanovas *et al.,* 2009). Conversely S-HJV, which is formed from the cleavage of HJV by transmembrane serine protease matriptase-2 encoded by the *TMPRSS6* gene, binds to and inhibits SMAD, reducing plasma hepcidin-25.

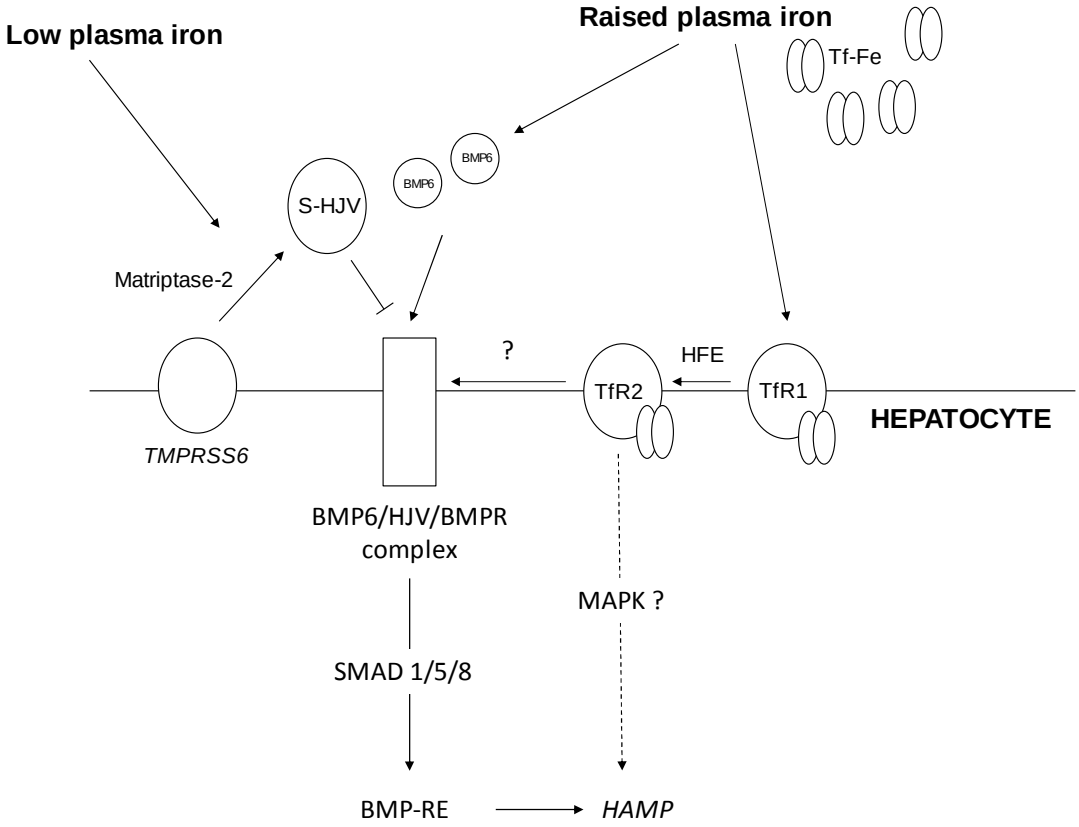


Figure 1-4. Schematic showing proposed roles of iron stores in hepcidin regulation (adapted from Babitt and Lin, 2010). (Tf-Fe: transferrin-iron complex; S-HJV: soluble haemojuvelin; HJV: haemojuvelin; BMP: bone morphogenetic protein; TfR1/2: transferrin receptor 1/2; MAPK: mitogen-activated protein kinase).

Down-regulation of hepcidin-25 synthesis causes a release of stored iron and an increase in dietary iron absorption. Circulating hepcidin-25 concentrations can be reduced by hypoxia, iron

deficiency, and increased erythropoietic activity. Hypoxia is a potent inhibitor of hepcidin-25 production and, although the exact mechanism has not been fully elucidated, it is thought to be related to the hypoxia inducing factor pathway (Liu *et al*., 2012). Regulation of hepcidin-25 production during iron-deficiency occurs via the BMP-6-HJV-SMAD signalling pathway described above, although the mechanism by which erythropoiesis regulates hepcidin-25 expression is poorly understood. However, recently erythroferrone, a member of the C1q/tumour necrosis factor-related protein family, which is expressed from erythroblasts in response to erythropoietin, has been suggested to be a key regulator (Kautz *et al.,* 2014; Pasricha *et al.,* 2016; Ganz *et al*., 2017). Other proteins such as growth/differentiation factor 15 (GDF15) and Twisted Gastrulation BMP Signalling Modulator 1 (TWSG1) are also released from erythroid pre-cursors, and are thought to inhibit the BMP-6-HJV-SMAD signalling pathway by an unknown mechanism (Hentze *et al.,* 2010).

1.3.3 Hepcidin Kinetics

Hepcidin-25 has been reported to bind to α-2-macroglobulin, and to a lesser extent albumin, with only 11 % of circulating hepcidin-25 non-protein bound (Peslova *et al.,* 2009); although another study has reported that less than 3 % of circulating hepcidin is protein-bound (Itkonen *et al.,* 2012). It is not clear whether binding of hepcidin-25 to plasma proteins affects its functional properties. The clearance of hepcidin-25 is thought to occur via degradation with FP-1 at its site of action, followed by excretion via the kidneys. It is also thought that non-protein bound hepcidin-25 may be filtered at the glomerulus, although fractional excretion rates of only up to 5 % have been reported (Kroot *et al.,* 2011). Nothing is known as to the degree of protein binding of the other hepcidin-25 isoforms, but hepcidin-20, -22, and -24 have all been identified in urine.

Concentrations of hepcidin-25 in the plasma and serum of healthy individuals have been extensively studied. In one of the largest studies (Galesloot *et al.,* 2011), median serum hepcidin-25 concentrations of 22 (95 % CI; 17–65) and 19 (95 % CI; 1–65) µg/L for males (N = 1066) and females (N = 882), respectively, were reported. This study measured hepcidin-25 using a competitive enzyme-linked immunosorbent assay (ELISA) and it should be borne in mind that there is considerable variability in the concentrations of hepcidin-25 measured depending on the methodology used (van der Vorm *et al.,* 2016). Despite this, these ranges are

comparable to others that have been reported using immunochemical and mass spectrometric methods (Busbridge *et al.,* 2009; Grebenchtchikov *et al.,* 2009; Kroot *et al.,* 2009a; Swinkels *et al.,* 2008).

Few studies have reported plasma or serum concentrations of other truncated isoforms of hepcidin-25. Mean serum hepcidin-20 concentrations in males (N = 706) and females (N = 871) of 9.4 and 9.6 µg/L, respectively, from a general population using Surface Enhanced Laser Desorption Ionisation-Mass Spectrometry (SELDI-MS) have been reported (Campostrini *et al*., 2012). However, hepcidin-20 was only detected (limit of detection: 1.6 µg/L) in some 50 % of samples. In 40 healthy volunteers, mean serum hepcidin-20, -22, and -24 concentrations of 2.6, 0.97, and 12.4 µg/L, respectively, have been reported (Addo *et al.,* 2016). Plasma and serum are in general used interchangeably, and broadly have shown to be comparable for hepcidin-25 (Butterfield *et al.,* 2010, Kobold *et al.,* 2008), although a bias of some 24 % for hepcidin-25 between human EDTA plasma and human serum has been reported (Lefebvre *et al.,* 2015). Nothing has been reported as to the comparability of plasma and serum for other isoforms of hepcidin.

1.4 Disorders of Iron Metabolism

Disorders of iron metabolism can be broadly divided into two main groups; (i) disorders of iron deficiency (which can be functional or absolute), and (ii) disorders of iron storage. Both groups of which are associated with either hepcidin-25 deficiency or excess.

1.4.1 Anaemia

It has been estimated that some 25 % of the population has anaemia, defined as a haemoglobin concentration < 13, and < 12 g/dL in males and females, respectively (McLean *et al.,* 2009). There are several causes of anaemia, but in infants and women of child bearing age, iron-deficiency (ID) is the most frequent cause and occurs in 2–3 %, and 5 % of individuals in these groups, respectively (Vermeulen & Vermeersch, 2012). After iron deficient anaemia (IDA), anaemia of chronic disease (ACD) is the second most prevalent form of anaemia, and is very common in hospitalised patients (Weiss & Goodnough, 2005). In one hospital review, 65 % of

patients developed anaemia and of these, ACD accounted for 57 % (Wong & Intragumtornchai, 2006).

Treatment of IDA and ACD is very different, and hence requires appropriate diagnosis. In patients with IDA, addressing the underlying cause of iron deficiency and treatment with iron supplements is the usual course of action. However, in patients with ACD, oral iron is poorly absorbed and treating the underlying cause of the infection is the priority. In individuals with ACD, hepcidin-25 is raised, primarily due to increased inflammatory cytokines (in-particular IL-6), preventing the absorption of dietary iron and cellular release. Individuals with ACD will have normal to raised ferritin concentrations, with appropriate iron stores, but low circulating plasma iron, and therefore reduced erythropoiesis. In contrast, in IDA, there are inadequate iron stores, for example caused by poor dietary intake, or excessive blood loss. These individuals would have low plasma hepcidin-25 concentrations. There are, however, individuals that have ACD with concomitant iron deficiency, and a suggested algorithm for the diagnosis of IDA, ACD/ID, and ACD is given in Figure 1-5. Anaemia is also common in patients with chronic kidney disease (CKD), in part due to reduced erythropoietin production, but also poor absorption of dietary iron and release from macrophages. This is thought to occur due to raised hepcidin-25 concentrations present from infection and inflammation, which is common in this group of patients, and through reduced hepcidin-25 excretion.

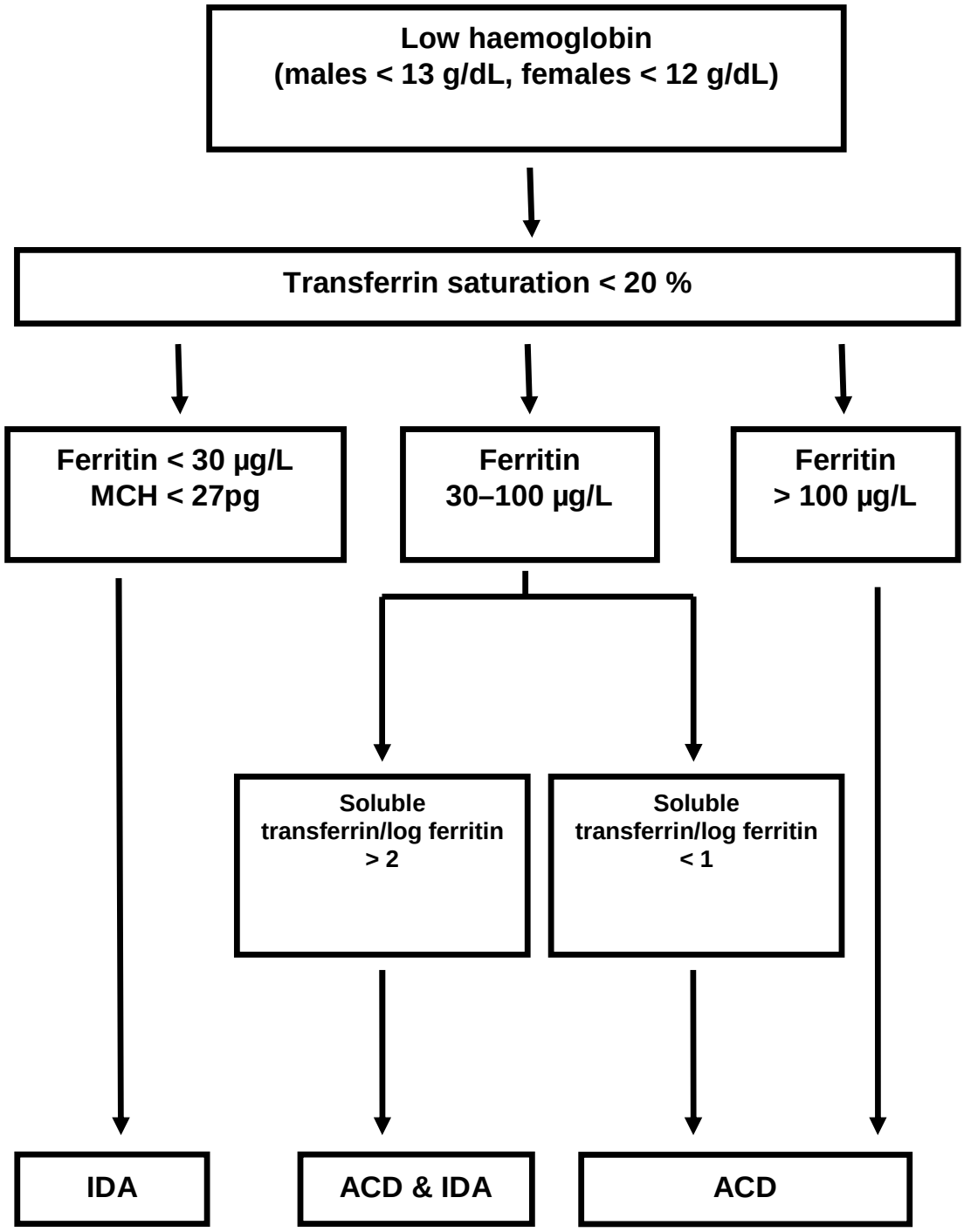


Figure 1-5. Suggested algorithm for the diagnosis of IDA, ACD/IDA, and ACD (modified from Weiss & Goodnough, 2005).

Another group of anaemias are the 'congenital iron-loading anaemias'. These include sickle cell anaemia (SCA), β-thalassemia, and congenital dyserythropoietic anaemia (CDA). In these anaemias, the diseased erythron dysregulates iron homeostasis via reduced hepcidin synthesis.

SCA is relatively common in individuals of African descent, and is characterised by mutations in the gene encoding for the β-globulin sub-unit of haemoglobin (HBB). SCA is when an individual has 2 copies of haemoglobin S (HbS), whereas individuals with HbS and haemoglobin C (HbC) have a milder form of SCA. During a 'sickle cell crisis', erythrocytes become sickle shaped and haemolyse, therefore individuals with SCA are characterised by a chronic haemolytic anaemia, increased erythropoiesis, and a chronic inflammatory state (Kroot *et al*., 2009b).

CDA is a rare disorder characterised by morphological abnormalities in erythroid pre-cursor cells. There are 3 main types, of which CDA II is the most common with approximately 370 cases identified worldwide (Iolascon *et al.,* 2012). CDA II is caused by a mutation in the *SEC23B* gene, which encodes the coat protein complex II protein, causing bi- and multi-nucleated erythroblasts, and therefore ineffective erythropoiesis, anaemia, and subsequent iron overload (Schwarz *et al.,* 2009; Casanovas *et al.,* 2011).

1.4.2 Iron overload and haemochromatosis

Iron overload can be caused by a number of factors one of which is liver disease such as alcoholic fatty liver disease or non-alcoholic fatty liver disease (NAFLD). In some populations (e.g. Asian) up to 40 % of all cases of iron overload have been attributed to non-haemochromatic causes (McDonald *et al.,* 2013), and NAFLD is one of the most common causes of liver disease in the USA where it has been estimated that some 75 % of all chronic liver disease cases are due to NAFLD (Hassan *et al.,* 2014). There are 4 main stages to NAFLD; (i) simple fatty liver (steatosis), (ii) non-alcoholic steatohepatitis (NASH), (iii) fibrosis, and (iv) cirrhosis. Individuals that are obese, have type 2 diabetes, high blood pressure, raised cholesterol, or smoke, are at increased risk of developing NAFLD. Individuals commonly present with fatigue together with metabolic syndrome (combination of diabetes, high blood pressure, and obesity), and increased hepatic iron is present - this is known as dysmetabolic iron overload syndrome (DIOS). Iron overload is associated with later stages of NAFLD, although the mechanism by which iron overload occurs is not clear (Britton *et al.,* 2016).

Hepcidin-25 has been reported to be raised in these individuals in response to the iron overload present (Boga *et al*., 2015).

Hereditary haemochromatosis (HH) is a common cause of iron overload in caucasian populations. Most individuals with HH have defects in the genes that encode proteins involved in the regulation of hepcidin-25 (i.e. HFE, TfR2, HJV). These individuals usually suffer from mild to moderate iron overload, and become symptomatic (and diagnosed) in adult-hood. However, in some rare forms of HH there are defects in the genes that directly encode for either FP-1 (*SLC40A1*) or hepcidin-25 (*HAMP*). When mutations in the FP-1 gene are present, it is known as 'ferroportin disease'. Individuals with mutations in this gene have severe iron overload that occurs at an earlier age than those individuals with mutations in the HFE gene, and are diagnosed in child hood or early adult-hood (20-30 years of age).

The most common form of HH is HFE-related, particularly a G to A transition at nucleoside 845 which leads to a cysteine to tyrosine substitution at amino acid position 282 (C282Y). Another common mutation is a C to G translation at nucleoside 187, leading to a histidine to aspartic acid substitution at amino acid position 63 (H63D). The substitution of cysteine to tyrosine at amino acid position 282 disrupts the interaction between HFE and β2-microglobulin, causing intracellular sequestration of HFE. Individuals with this genotype are partially responsive to acute/chronic iron loading, but have markedly reduced synthesis of hepcidin-25, which is inappropriate to the overload of iron present. Conversely, the C to G transition at nucleoside 187 (H63D) does not disrupt binding of HFE with β_2-microglobulin (Feder *et al*. 1997). Therefore, this mutation has little clinical significance with regards to iron overload.

The prevalence of C282Y homozygosity has been suggested to be 1 in 200 individuals, although the penetrance of this genotype is only 5 % (Pietrangelo, 2015; Rossi & Jeffrey, 2004). C282Y heterozygosity alone is rarely associated with iron overload and other factors, such as diabetes, liver diseases, and alcohol abuse have been proposed as potential causes of iron overload in individuals with this genotype, although this is controversial (Aguilar-Martinez *et al.,* 2011). The H63D polymorphism is much more prevalent (1 in 7 individuals) than C282Y homozygosity but it has little clinical penetrance, and is usually associated with other conditions of iron overload for example diabetes (Pietrangelo 2015; Steinberg *et al.,* 2001).

1.5 Clinical Applications of Hepcidin Measurement

Several potential diagnostic and therapeutic applications for hepcidin-25 measurement have been suggested, although it is not yet clear whether there is any clinical rationale for the measurement of hepcidin-20, -22, or -24.

One area that shows promise for hepcidin-25 measurement is the differential diagnosis of IDA from ACD since hepcidin-25 is low in IDA but inappropriately raised in ACD (van Santen *et al.,* 2011). Currently there is no single laboratory test that can be used to differentiate between these two conditions, instead the results from a number of biochemical tests (e.g. ferritin, transferrin saturation) are reviewed in combination to aid diagnosis.

Another promising application for hepcidin-25 measurement is in the treatment of anaemia in patients with CKD. In this group of patient's anaemia is common, partly due to inadequate production of EPO, and therefore patients are commonly treated with erythropoietin stimulating agents (ESAs) or iron supplementation. However, some of these patients do not respond to treatment with iron, which in part maybe due to raised plasma hepcidin-25 concentrations (most likely due to chronic inflammation) inhibiting duodenal iron uptake and iron release from cellular stores (Konz *et al.,* 2014). Clinically, measurement of hepcidin-25 in these patients would help identify those that would respond from oral iron supplementation.

As regards to therapeutic applications, there are a number of agents that are being developed that manipulate the mechanisms involved in hepcidin-25 production (Boyce *et al*., 2016; Liu *et al*., 2016; Poli *et al.,* 2014,). In this situation measurement of plasma hepcidin-25 could be of use to assess the effectiveness of these agents.

1.6 Methodology

1.6.1 Mass spectrometry

Mass spectrometry (MS) is the gas phase separation of ionized atomic or molecular species according to their mass-to-charge ratio (*m/z*). Following ionisation of an analyte, a characteristic ion that represents the intact atom/molecule or a group of ions that represent fragments of the ionized species are formed. These ions are then separated by manipulating magnetic or electrostatic fields in a high vacuum, and detected using an electron multiplier tube. The plot of their relative abundance against the *m/z* of each ion is a mass spectrum.

The basic components of a mass spectrometer are: (i) sample introduction system, (ii) an ion source, (iii) mass analyser, (iv) a detector (typically an electron multiplier tube), and (v) a data collection system (Flanagan *et al*., 2007). There are a number of different types of mass analysers available, primarily; (i) sector/double focusing, (ii) time of flight (TOF), (iii) quadrupole, (iv) quadrupole ion trap, (v) fourier-transform ion cyclotron resonance, and (vi) the recently introduced Orbitrap mass analyser. Two important parameters used in mass spectrometry that define instrument, and method related performance are 'resolving power' and 'resolution', respectively. Resolving power is the ability to distinguish two adjacent ions of equal intensity, and the IUPAC definition (Murray *et al*., 2013) is –

"For two peaks of equal height with masses m_1 and m_2, when there is overlap between the two peaks to a stated percentage of either peak height (10 % is recommended), then the resolving power is defined as: $m_1/(m_1 - m_2)$"

Whereas resolution is a function of both the ion width and mass being measured, and the IUPAC definition (Murray *et al*., 2013) is –

"For a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution may be expressed as: $m/\Delta m$. Where Δm is the width of the peak at a height which is a specified fraction of the maximum peak height (for example, 50 %)."

Resolving power is important as the greater the resolving power, the greater the ability to differentiate two ions of similar mass, and therefore improve selectivity. Typically, quadrupole instruments have a resolving power of broadly 2000 FWHM (at *m/z* 200) and an ion peak width of 0.6 atomic mass units (amu), hence such instruments are referred to as 'low resolution' (LR-MS). TOF instruments typically have a resolving power of around 15,000 FWHM (at *m/z* 400), and Orbitrap instruments of 140,000 FWHM (at *m/z* 200), and are known as 'high resolution' (0.1 ≥ amu, HR-MS, Rochat *et al*., 2012).

1.6.2 Hepcidin methodology

Since the discovery of hepcidin-25, several methods have been published for its quantitation in plasma, serum and urine. These methods broadly fall into two categories, (i) immunochemical, and (ii) mass spectrometric. There is no 'gold-standard' assay for the quantitation of hepcidin-25

or *N*-truncated analogues, nor any external quality assurance scheme or external quality control material commercially available. Therefore, much variability between mass spectrometry and immunochemical based methods has been reported, and even much variability within each technique (Kroot *et al.,* 2009; Kroot *et al.,* 2012; van der Vorm *et al.,* 2016). The introduction of a commercially available isotopically labelled hepcidin-25 for use as an internal standard has the potential to greatly improve the accuracy and precision of mass spectrometric hepcidin-25 assays. Of course, this still depends on the sample preparation and detection conditions being fully optimised, and the accurate preparation of calibration solutions.

1.6.2.1 Immunochemical assays

Immunochemical assays are widely used in routine clinical laboratories as they can be easily automated and do not require highly trained staff. For hepcidin-25, competitive enzyme linked immunosorbent assays (cELISA, Kroot *et al.,* 2010; Ganz *et al.,* 2008; Koliaraki *et al.,* 2009; Schwarz *et al.,* 2011), competitive radioimmunoassays (cRIA, Busbridge *et al.,* 2009; Grebenchtchikov *et al.,* 2009) and a 2-site ELISA have been reported (Butterfiled *et al.,* 2010). However, the development of a reliable immunochemical method for the measurement of hepcidin-25 has been complicated by difficulties in generating specific hepcidin-25 antibodies in animals such as rabbits. It is likely that currently available immunochemical assays will detect all known isoforms of hepcidin (e.g. hepcidin-20, -22, and -24) to some degree, and not just hepcidin-25.

1.6.2.2 Matrix Assisted and Surface Enhanced Laser Desorption Ionisation

In Matrix Assisted Laser Desorption Ionisation (MALDI) the sample is mixed with an organic compound (e.g. 2-mercapto-nezothiazole) in a suitable solvent. This mixture is then spotted onto a sample planchet, where the solvent is evaporated leaving a crystalised matrix. A UV laser, most typically based on nitrogen, is focused onto the sample in short bursts, which induces desorption and ionization of the analyte and matrix. The ions formed are then analysed using a time of flight mass spectrometer (TOF-MS). MALDI-TOF-MS is a ‘soft’ ionization technique with little, if any fragmentation of the protonated molecule (Watson and Sparkman, 2007), which makes it a useful technique for determining the molecular mass of peptides, proteins or polymers. Therefore, it is widely used for qualitative analysis, although it has been

used for the quantification of hepcidin-25 in serum or plasma (Anderson *et al.,* 2011; Kroot *et al.,* 2010) and in urine (Anderson *et al.,* 2010; Bansal *et al.,* 2009a; Gay *et al.,* 2010).

Surface Enhanced Laser Desorption/Ionisation (SELDI) is a variant of MALDI where the analyte is bound to a surface that has incorporated chemistry, i.e. hydrophobic, cationic, anionic, metal ion presenting or hydrophilic surfaces. Once the sample has been dispensed onto the surface it is incubated after a washing step where any proteins or contaminants are that are not retained by the surface chemistry are removed. One advantage of this approach is that components such as salts or detergents that may interfere with analysis are removed during the washing step (Seibert *et al.,* 2004). SELDI-TOF was the first MS based method described for the semi-quantitative measurement of hepcidin-25 in human urine and plasma (Tomosugi *et al.,* 2006). Several fully quantitative SELDI-MS methods have since been reported for the measurement of hepcidin-25 in serum (Swinkels *et al.,* 2008; Ward *et al.,* 2008) and urine (Bozzini *et al.,* 2008; Swinkels *et al.,* 2008; Altamura *et al.,* 2009).

A partial disadvantage of TOF mass spectrometers is that they operate at a constant resolving power. This means that smaller differences between two *m/z* values can be separated at the low end of the instruments range (e.g. *m/z* 200) than at the high end (e.g. *m/z* 1,000), and importantly, resolution increases with increasing *m/z* values. Therefore, when analysing peptides that typically have a high *m/z* it may not be possible to completely resolve ions of different masses or isotopes. Thereby, there is potential for accurate and precise quantitation to be compromised, should the assay not be fully optimised and validated. Furthermore, together with limited sample pre-treatment opportunities, and no LC, prior to analysis by MS, analyte specificity maybe compromised (Anderson *et al.,* 2010).

1.6.2.3 Liquid Chromatography-Mass Spectrometry [LC-MS(/MS)]

An increasing number of methods have been reported for the measurement of hepcidin-25 using liquid chromatography-mass spectrometry (LC-MS). Most reported methods use LC coupled with a triple quadrupole mass spectrometer (TQMS), and the data acquired using selected/multiple reaction monitoring (S/MRM). In this technique, following appropriate sample preparation, analytes are separated by liquid chromatography and a pre-selected precursor ion undergoes collision induced dissociation under controlled conditions, and the formation of defined product ion(s) is monitored. The first liquid chromatography tandem mass spectrometry

(LC-MS/MS) based method for hepcidin-25 analysis to be reported was by Murphy *et al.,* (2007). This method used calcitonin gene-related peptide as an internal standard, which is not ideal due to its different physiochemical properties to that of hepcidin-25. Despite this, a lower limit of quantitation of 1 µg/L was reported, and accuracy and precision were deemed acceptable. Several other LC-MS/MS based methods using a TQMS for the quantification of hepcidin-25 in either serum (Murao *et al.,* 2007; Kobold *et al.,* 2008; Li *et al.,* 2009; Hwang *et al.,* 2011; Wolff *et al.,* 2013; Delaby *et al*., 2014, Lefebvre *et al.,* 2015) or urine (Hwang *et al.,* 2011; Wolff *et al.,* 2013) have been since been published.

One concern in using TQMS for peptide analysis is that usually a product ion(s) of one charge state, and one isotope is monitored, however there maybe sample-to-sample variability in the isotope and charge state distribution, thereby compromising accurate quantitation. Furthermore, in LC-MS/MS, the analyte should fragment appropriately (i.e. to a few but not too many ions). An LC-MS/MS method for the measurement of hepcidin-25 in plasma has also been reported using an ion-trap MS (Bansal *et al.,* 2010).

Traditionally high resolution-mass spectrometry (HR-MS) has been used predominantly for qualitative analysis, such as drug metabolite identification, and proteomics. However, in recent years with the introduction of Orbitrap mass analysers from ThermoFisher Scientific in 2005, HR-MS is being increasingly used for quantitative analysis in clinical settings. Orbitrap instruments have high resolution capabilities, and maximal resolving powers of up to 280,000 FWHM (*m/z* 200) in the most advanced model currently available (Q-Exactive Plus, ThermoFisher Scientific). Furthermore, they have an extended mass range of up to 6000 *m/z*. Instruments are now available that include a quadrupole as well as an Orbitrap (Q-Exactive), that allow data to be collected not only in full scan mode, but also for ions to be fragmented and all products collected (parallel reaction monitoring). HR-MS has several advantages compared to LR-MS, for example: (i) if data is acquired in full scan mode, the collision energy for each analyte does not have to be optimised as with LC-MS/MS, (ii) HR-MS allows full scan data to be acquired, and for all charge states and isotopes to be monitored, (iii) when data is acquired in full-scan mode it can be retrospectively audited for compounds not initially targeted, and (iv) almost all pre-cursor ions entering the instrument can be spectrometrically resolved (depending on resolving power used). To date, two methods have been published for the measurement of hepcidin-25 using HR-MS. The first was reported by Rochat *et al.,* (2013) where an Exactive

Plus Orbitrap (ThermoFisher Scientific) was used to acquire data in full scan mode using a single charge state for quantitation, but only the 3 most abundant isotopes of that charge state were monitored. Li *et al.,* (2014) reported a method for the measurement of hepcidin-25 in mice and cynomolgus monkey serum using a Q-Exactive Orbitrap (ThermoFisher Scientific), with data acquired in parallel reaction monitoring (PRM) mode, but again only a single charge state was used for quantitation.

Regarding sample preparation prior to detection with LC-MS(MS), solid phase extraction (SPE) is most widely used for hepcidin-25 measurement (Murphy *et al.,* 2007; Li *et al.,* 2009; Wolff *et al.,* 2013; Li *et al.,* 2014; Lefebvre *et al.,* 2015). Other sample preparation techniques that have been used include protein precipitation (PPT), ultrafiltration, and the use of magnetic nanoparticles prior to LC-MS/MS. The preference for using SPE is, in part, because SPE allows concentration of the analyte onto the sorbent, and by introducing wash steps of the SPE cartridge with appropriate solvents, compounds that may interfere with accurate quantification can be removed prior to elution of the desired analyte. Furthermore, 96-well plates are available, thereby allowing relatively high throughput.

1.6.2.4 Inductively Coupled Plasma mass spectrometry (ICP-MS)

Inductively Coupled Plasma-Mass Spectrometry, unlike LC-MS/MS, is an inorganic technique. The advantage of this technique is that ion-suppression is less of a concern compared to LC-MS(/MS) analysis, and that calibration solutions containing the specific analyte are not required, instead solutions containing the element to be monitored at known concentrations are used. Hepcidin-25 is composed of 9 amino acids that contain sulphur (8 cysteines and 1 methionine), and with this in mind a method has been published for the measurement of hepcidin-25 in human urine monitoring ^{32}S (Konz *et al.,* 2011). In this method samples were extracted using SPE and then analysed by capillary liquid chromatography coupled to ICP-MS. A solution of ^{34}S was continuously pumped and mixed with the column effluent prior to being introduced into the ICP-MS as an internal standard. The ratio of ^{32}S:^{34}S was used to quantify ^{32}S. The reported limit of detection was 7 µg/L for ^{32}S, which corresponded to 70 µg/L of hepcidin-25; however, this was insufficient to measure hepcidin-25 in urine samples from healthy volunteers. Konz *et al*. (2012) exploited the binding of Cu^{2+}ions at the *N*-terminus of hepcidin-25 for the measurement of hepcidin-25 in human serum. In this technique serum samples from healthy volunteers and

those with Parkinson's disease were incubated with a solution of copper in 10 mM ammonium acetate. Samples were analysed by liquid chromatography coupled to ICP-MS with a solution of ^{65}Cu introduced post-column. The ^{63}Cu:^{65}Cu ratio was used to quantify ^{63}Cu as a surrogate for hepcidin-25, and an LLoQ of 1.8 µg/L for hepcidin-25 was achieved.

The use of ICP-MS for the quantitation of hepcidin-25 is not practical in a clinical setting because these instruments are infrequently used in routine clinical laboratories, except for those that offer a trace elements/metals service.

1.7 Aim and objectives of thesis

The aim of this work will be to define what role measurement of hepcidin isoforms have in a clinical setting, and the hypothesis that hepcidin-25 measurement has a clinical role. The objectives undertaken to achieve this will be: (i) develop a LC-HR-MS based method for serum hepcidin-20, -22, -24, and -25; (ii) validate the method, including a method comparison with samples analysed by existing immunoassay and LC-MS/MS assays; (iii) define a method based reference range for all hepcidin isoforms using samples from healthy volunteers; (iv) investigate analyte stability in different matrices under a range of storage conditions (i.e. room temperature, 2-8 °C, and -20 °C); (v) investigate hepcidin isoform concentrations in patients with various stages of CKD, in those undergoing dialysis as well as those not requiring haemodialysis, correlating hepcidin concentrations with traditional markers of iron status (e.g. ferritin, transferrin saturation); and (vi) investigate concentrations of hepcidin isoforms in patients with iron disorders including haemochromatosis, IDA, ACD, ID, and SCA.

Chapter 2 Method Development

2.1 Introduction

When developing any method for the measurement of a given analyte, optimisation of the sample preparation technique and detection method is essential in order to ensure optimum sensitivity, and accurate/precise quantitation is achieved. This can be time consuming and is influenced by the chemical and physical properties of the analyte(s). In general, when developing an LC-MS based method, the MS parameters (detection) are broadly optimised first, followed by the LC conditions (chromatographic separation), and then finally suitable sample preparation techniques are investigated.

The method development undertaken here will use a Q-Exactive MS, and once developed, this instrument will continue to be used. This is because: (i) it has high resolution capabilities (ii), can be operated in full-scan mode, allowing acquisition of all charge states and isotopes for post-acquisition data processing (iii), it has a quadrupole as well as an orbitrap mass analyser, therefore allowing parallel reaction monitoring if required and (iv), this instrument is not routinely used within the laboratory.

The aims of this chapter are as follows:

- Optimise mass spectrometric, and liquid chromatographic conditions for all available hepcidin isoforms
- Investigate protein precipitation, immunocapture and solid phase extraction as suitable sample preparation techniques
- Fully optimise chosen sample preparation technique, ready for validation

2.2 Materials and methods

2.2.1 Materials

Human hepcidin-20, -22, -24, -25, and isotopically labelled hepcidin-25 [($^{13}C_8$,$^{15}N_3$)-hepcidin-25; hepcidin-ISTD] were from Peptides International (Kentucky, USA). Amino acid sequences ($MW_{monoisotopic}$) for hepcidin-20, -22, -24, and -25 were: ICIFCCGCCHRSKCGMCCKT (2189.771), FPICIFCCGCCHRSKCGMCCKT (2433.892), THFPICIFCCGCCHRSKCGMCCKT (2671.999), and DTHFPICIFCCGCCHRSKCGMCCKT (2787.026), respectively. The $MW_{monoisotopic}$ for hepcidin-ISTD was 2813.114. The purity of all compounds was greater than 95.9 %, however, the net peptide content ranged from 65.9–84.1 % (100 % for hepcidin-ISTD). All were supplied as trifluoroacetate salts. Heavy (quadruple) charcoal stripped human serum (SHS) was from Sera Laboratories Ltd (West Sussex, UK). All reagents used were from Sigma Aldrich (Poole, UK) and were of either ACS reagent or HPLC grade. Water was deionised (> 12 mΩ, Elga, Marlow, UK). All analytical LC columns were purchased from Hichrom (Theale, UK). Disposal Automated Research Tips (D.A.R.T) were pre-bound with anti-hepcidin-25 polyclonal antibodies (Pierce, ThermoFisher Scientific, Rockford USA) by ThermoFisher Scientific (Tempe, USA). Protein LoBind 0.5 and 1.5 mL tubes, and 500 µL 96-deep-well plates were from Eppendorf (Stevenage, UK). pH testing strips were from VWR (Lutterworth, UK). HBS-EP buffer containing; 0.01 mol/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 0.15 mol/L sodium chloride, 3 mmol/L EDTA, and 0.005 % (*v/v*) surfactant polysorbate20 (P20) was from GE Healthcare Life Sciences (Little Chalfont, UK). Polysorbate20, sodium dodecyl sulfate (SDS), and Triton X-100 (all > 99 %) were from Sigma Aldrich (Poole, UK). Oasis µElution 96-well SPE plates (30 µm particle size, 2 mg packing per well) were obtained from Waters (Elstree, UK).

Unless otherwise stated hepcidin-20, -22, -24, -25 and hepcidin-ISTD were added to SHS at a final concentration of 100 µg/L for developmental work. Portions (1.0 mL) were stored in 1.5 mL LoBind tubes at -20 °C until required.

2.2.2 Instrumentation

An Aria Transcend LX-II system (ThermoFisher Scientific, San Jose, CA) consisting of 4 Accela 1250 high-pressure quaternary pumps, valve interface module, and CTC PAL autosampler was

used with a Q-Exactive Orbitrap high resolution MS (ThermoFisher Scientific, Bremen, Germany). The autosampler tray was maintained at 10 °C. Instrument control was performed using Xcalibur software (version 3.1; ThermoFisher Scientific). A Finnpipette Novus 12 channel pipette (Thermo Fisher Scientific, Bremen, Germany) was used for immunocapture. A Waters extraction manifold for 96-well plates was used for solid phase extraction. Theoretical isotope patterns, *m/z*, and product ions were calculated using Pinpoint software (v 1.1, ThermoFisher Scientific, San Jose, USA). Final MS conditions were as follows; spray voltage 4.0 kV; temperatures: vaporiser 150 ºC; capillary 350 ºC; auxiliary, sheath, and sweep gases 10, 35 and 0 (arbitrary units) respectively, S-lens voltage 90 V. The C-trap capacity was set at 3 x 10^6 charges, and maximum injection time was 200 ms. Full-scan MS data were acquired using a resolving power of 70,000, defined as full width at half maximum at *m/z* 200, with a scan range of 400–1,000 *m/z*.

For all post-acquisition data processing, peak areas were generated by filtering full-scan data using a mass extraction window of ± 10 ppm based on theoretical *m/z* values of the five most abundant isotopes of the $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$ (Table 2.1). External mass calibration was carried out using a positive mass calibration solution (ThermoFisher Scientific, Bremen). Peak area ratios (hepcidin-20, -22, -24, and -25 to hepcidin-ISTD) were measured and used to construct calibration curves ($1/x^2$ weighting, not forced through zero).

2.2.3 Extraction yield, matrix effects, and process efficiency

The extraction yield, matrix effects, and process efficiency were assessed per accepted strategies (Matuszewski *et al.,* 2003). All hepcidins were added; (A) to the appropriate extraction solution at a concentration corrected for sample concentration, (B) after, or (C) before the extraction procedure to the appropriate biological matrix.

This approach allows the matrix effect (ME), extraction recovery (RE) of the extraction procedure, and overall "process efficiency" (PE) to be calculated as follows:

ME (%)	=	B/A	x	100
RE (%)	=	C/B	x	100
PE (%)	=	C/A	x	100

For matrix effects a value greater than 100 % may suggest ion enhancement, whereas a value less than 100 % may suggest ion suppression. Extraction recovery is the absolute amount (expressed as a percentage) of a compound that has been extracted from the sample using a

given procedure not taking into account matrix effects. Process efficiency is the overall percentage of compound that has been extracted and takes into account the degree of matrix effect. The ME, RE and PE, are in part calculated by comparison to a solution containing all the analytes that has not been through the extraction process, but directly injected onto the LC-HR-MS.

2.3 Optimising Mass Spectrometry Conditions

In mass spectrometry, optimisation of the signal obtained for a given analyte is generally undertaken by direct infusion of the analyte via a syringe pump into the MS at a flow-rate and in a solution comparable to that used in the final LC method. From this, a mass spectrum is obtained, which is a plot of the relative abundances of ions as a function of their *m/z*. Due to the large size of peptides and proteins, when analysed using electrospray ionisation (ESI) they become multiply charged, and several protonated molecules of varying charge states are present in the mass spectrum.

To investigate the mass spectrum of all hepcidins, separate 200 mg/L aqueous solutions containing 0.1 % (*v/v*) formic acid of each hepcidin was directly infused into the MS using heated electrospray ionisation (HESI) in positive mode and full scan data acquired (100–1,500 *m/z*). Theoretical *m/z* values of the five most abundant isotopes of the $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$, are shown in Table 2.1.

Hepcidin-20, and hepcidin-22 gave the greatest response, followed by hepcidin-24, and then hepcidin-25, and hepcidin-ISTD. The response of hepcidin-20 was some three times that of hepcidin-25 and hepcidin-ISTD at the concentration investigated. This may in part be due to poor ionisation of hepcidin-25 since the amino acid at the *N*-terminus of hepcidin-25 is aspartic acid, which is negatively charged and not able to accept a positive charge, whereas the amino acids at the *N*-terminus of hepcidin-20, -22, and-24 are isoleucine, phenylalanine, and tyrosine respectively, which are neutrally charged, hence may be able to accept some charge when positively ionised. For most analytes, $[M+2H]^{2+}$ to $[M+6H]^{6+}$ were present, and for all analytes except hepcidin-20, the most abundant ions observed were $[M+4H]^{4+}$ and $[M+5H]^{5+}$ (Figure 2-1, Figure 2-2). For hepcidin-20 however, the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ were most abundant (Figure 2-1). A magnified mass spectrum of the $[M+5H]^{5+}$ of hepcidin-25, showing the theoretical *m/z* of

ten isotopes together with the contribution of each isotope to the overall charge state is given in Figure 2-3. When infusing each separate hepcidin isoform, neither hepcidin-20, -22, -24, or -25 were present when they should not have been (which is in-keeping with the high purity stated on the analytical data sheets), and shows a lack of in-source molecular degradation. However, when reviewing the mass spectrum of hepcidin-20, ions corresponding to the theoretical masses of the $[M+4H]^{4+}$ charge state of hepcidin-19 were observed (*m/z* 520.1790, 520.4300, 520.6790), although the relative proportion of these ions to the corresponding charge state of hepcidin-20, were extremely low (< 1 %).

Table 2.1. Theoretical masses of the top five isotopes of the $[M+3]^{3+}$, $[M+4]^{4+}$, and $[M+5]^{5+}$ of hepcidin-20, -22, -24, -25, and hepcidin-ISTD.

	Charge state		
	$[M+3]^{3+}$	**$[M+4]^{4+}$**	**$[M+5]^{5+}$**
Hepcidin-20	731.2651 731.5982 730.9310 731.9316 732.2647	548.7007 548.9504 548.4500 549.2005 549.4504	439.1620 439.3618 439.5619 438.9615 439.7617
Hepcidin-22	812.6389 812.9721 812.3047 813.3055 813.6388	609.7310 609.9809 609.4803 610.2310 610.4809	487.9862 488.1862 487.7857 488.3862 488.5862
Hepcidin-24	892.3411 892.0078 892.6746 891.6735 893.0079	669.5076 669.2576 669.7578 669.0070 670.0077	535.8076 535.6075 536.0076 535.4070 536.2076
Hepcidin-25	930.6835 930.3501 931.0169 930.0159 931.3503	698.5145 698.0143 698.2644 697.7637 698.7645	559.0131 558.8130 558.6130 558.4124 559.2131
Hepcidin-ISTD	937.7006 937.3672 938.0341 937.0330 938.3674	703.5273 703.2772 703.7774 703.0266 704.0274	563.0233 562.8232 563.2234 562.6227 563.4233

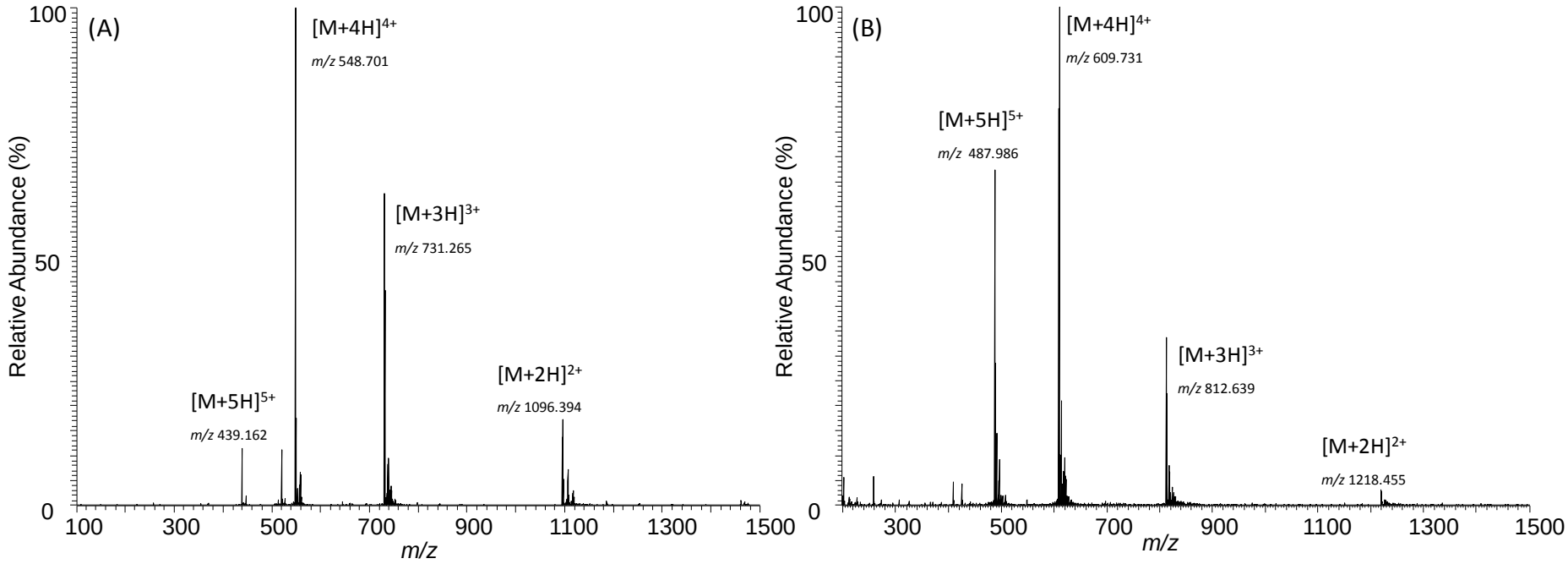


Figure 2-1. Mass spectra of (A) hepcidin-20, and (B) hepcidin-22 following direct infusion of separate 200 mg/L aqueous solutions containing 0.1 % (*v/v*) formic acid into the MS. Charge state and theoretical monoisotopic *m/z* are shown.

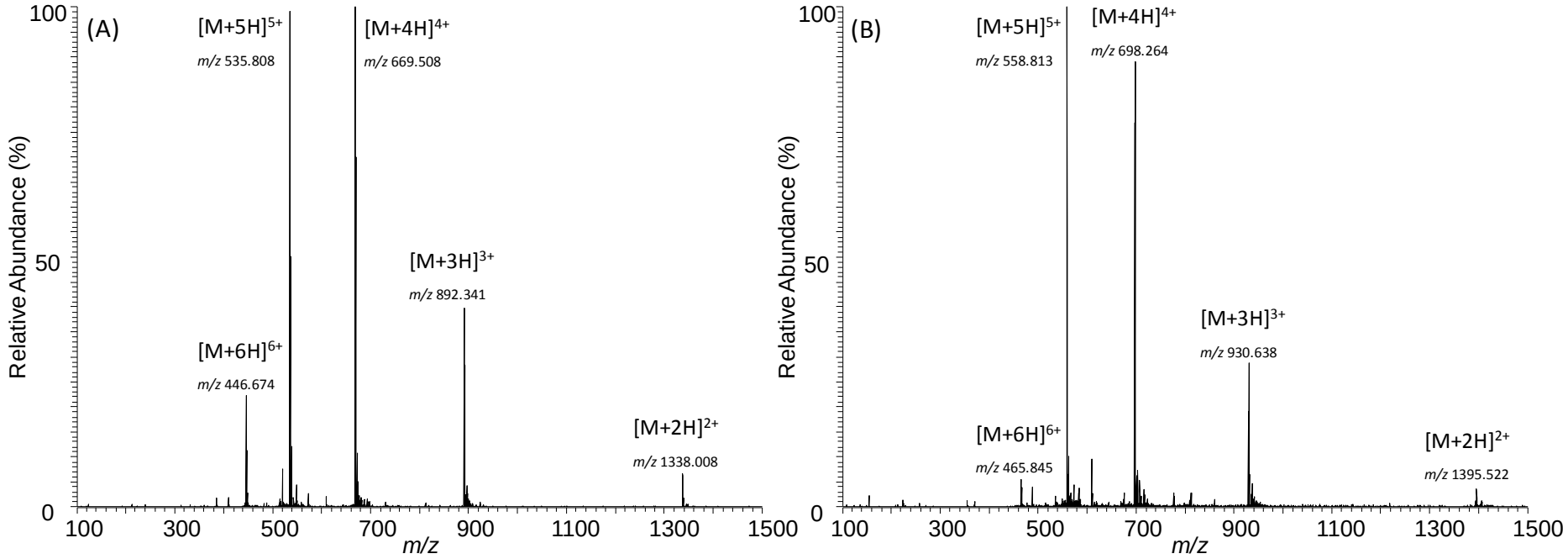


Figure 2-2 Mass spectra of (A) hepcidin-24, and (B) hepcidin-25, following direct infusion of separate 200 mg/L aqueous solutions containing 0.1 % (*v/v*) formic acid into the MS. Charge state and theoretical monoisotopic *m/z* are shown.

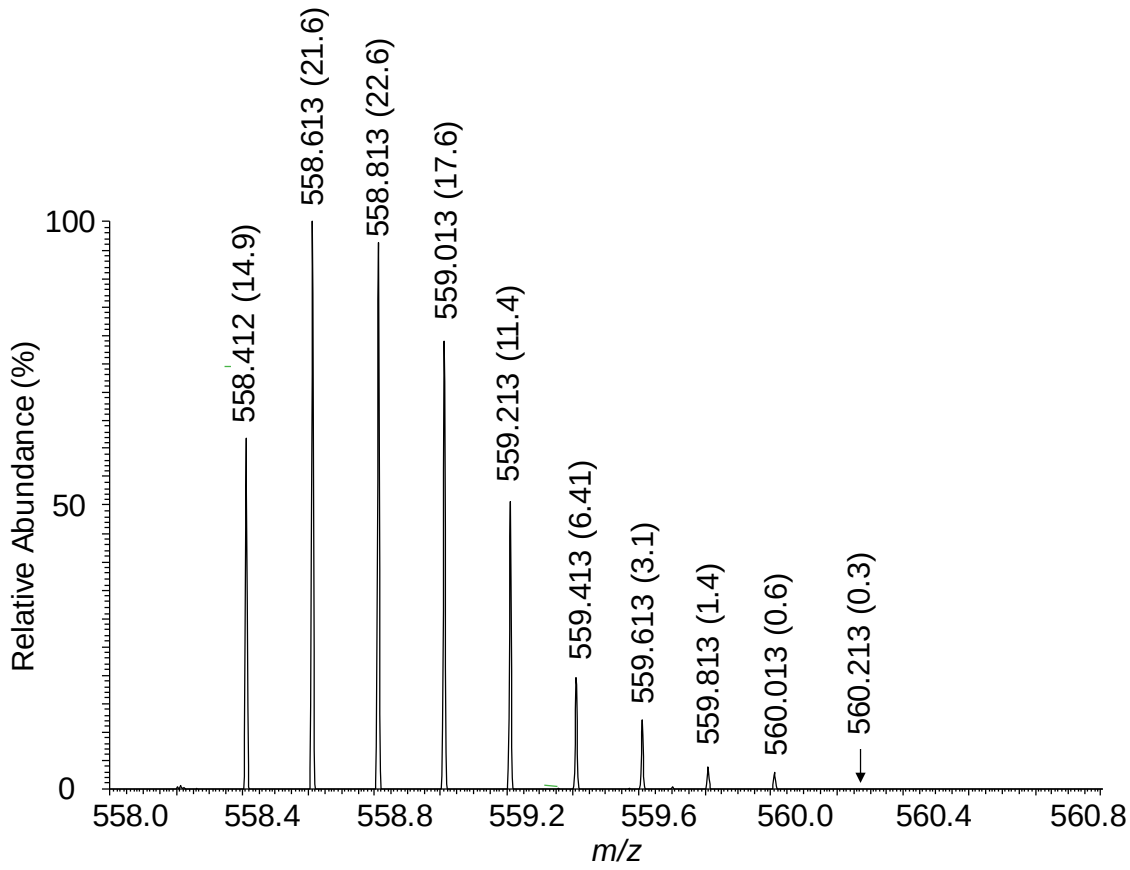


Figure 2-3. Magnified mass spectrum of the $[M+5H]^{5+}$ ion of hepcidin-25 (resolving power 140,000 defined as FWHM at *m/z* 200; figure in parentheses: percentage contribution of isotope to total charge).

When peptides are fragmented, the product ion scan contains 'b' and 'y' ions. Product ions that extend from the *N*-terminus are known as b ions, and those that extend from the *C*-terminus are known as y ions. These b and y ions can also be multiply charged, as can the precursor ion.

Fragmentation of the $[M+5H]^{5+}$ of hepcidin-25 was investigated at a collision energy of 20 eV. The most abundant product ion was the $y19^{3+}$ (*m/z* 693.236) ion, with many other b and y ions present, but no product ion of any significant abundance (Figure 2-4). It is not surprising that some product ions of hepcidin-25 were hepcidin-20 (y_{20}^{3+}), -21 (y_{21}^{3+}), and -22 (y_{22}^{3+}), which were themselves multiply charged. Increasing the collision energy to 30 eV completely fragmented the precursor ion, resulting in many small product ions of no significant abundance. The product ions of all hepcidin-20, -22, and -24 were also investigated following collision

induced dissociation at 20 and 30 eV, the mass spectra obtained were all similar, with the y19 ion being common to all.

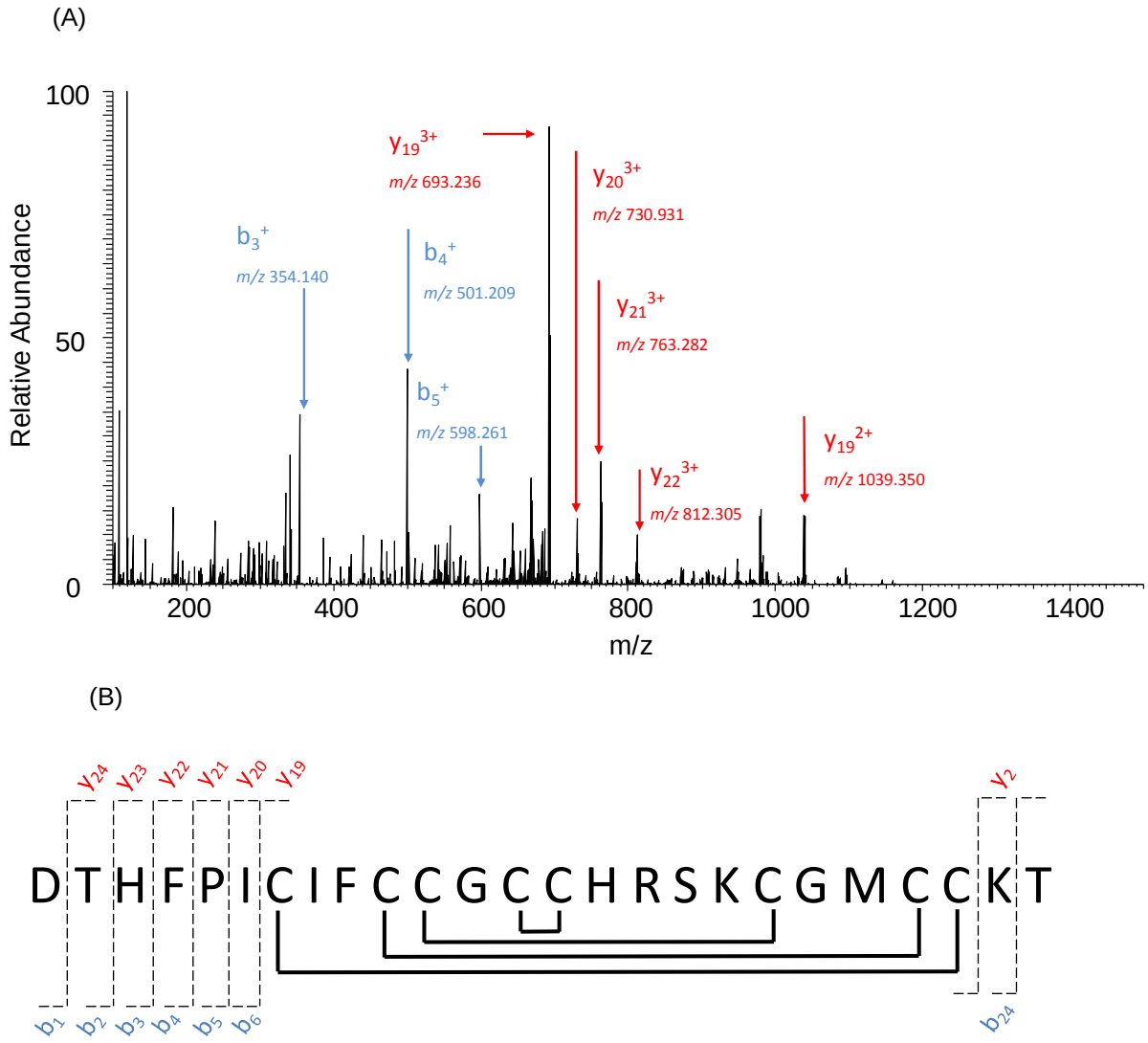


Figure 2-4. (A) Product ion scan of the $[M+5H]^{5+}$ of hepcidin-25 at 20 eV, and (B) amino acid sequence of hepcidin-25 indicating b and y ions.

Modifying the charge state of peptides or proteins is undertaken in some instances to; (i) concentrate the signal into fewer charge states, (ii) simplify spectra, and (iii) to improve signal-to-noise-ratios (Krusemark *et al.,* 2009). To investigate this for those analytes studied here, 3–nitrobenzyl alcohol (3-NBA), a common additive used to modify charge state, was added (0.1 % *v/v*) to the infusion solution of each analyte. Mass spectrums obtained are given in Figure 2-5 and Figure 2-6. The addition of 3-NBA markedly altered the charge state distribution of all

hepcidins. However, it did not concentrate the signal into a single charge state, which would be desirable as it may help to improve the LLoQ. Increasing the concentration of 3-NBA from 0.1 to 1.0 % (*v/v*) did not alter the charge state further. The use of 3-NBA as an additive to modify the charge state of hepcidin-25 has been reported (Rochat *et al.,* 2013) when using an Exactive Plus Orbitrap analyser (ThermoFisher Scientific, Bremen), where, a similar charge state distribution was reported to that identified here when using 0.1 % (*v/v*) 3-NBA. Since the addition of 3-NBA did not concentrate the signal of any analyte into a single charge state, it was considered to be of no benefit and was not investigated any further.

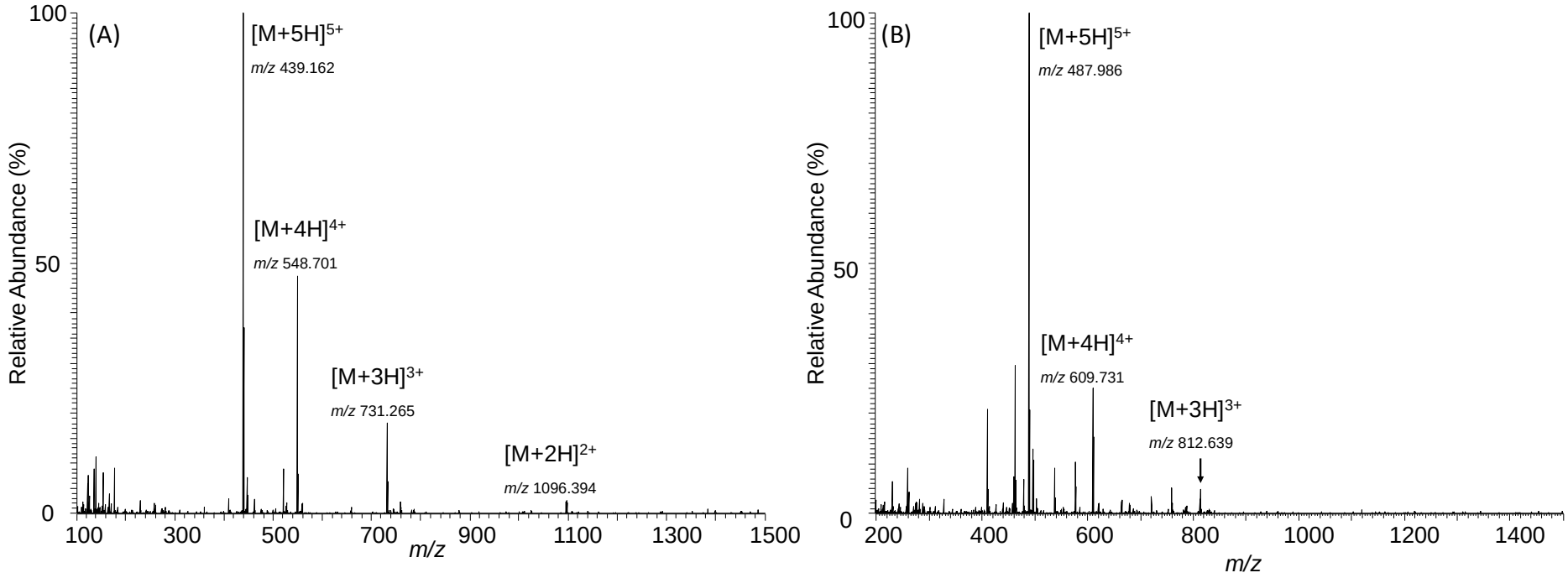


Figure 2-5. Mass spectra of (A) hepcidin-20, and (B) hepcidin-22, following direct infusion of separate 200 mg/L aqueous solutions containing 0.1 % (*v/v*) 3-NBA into the MS. Charge state and theoretical monoisotopic *m/z* are shown.

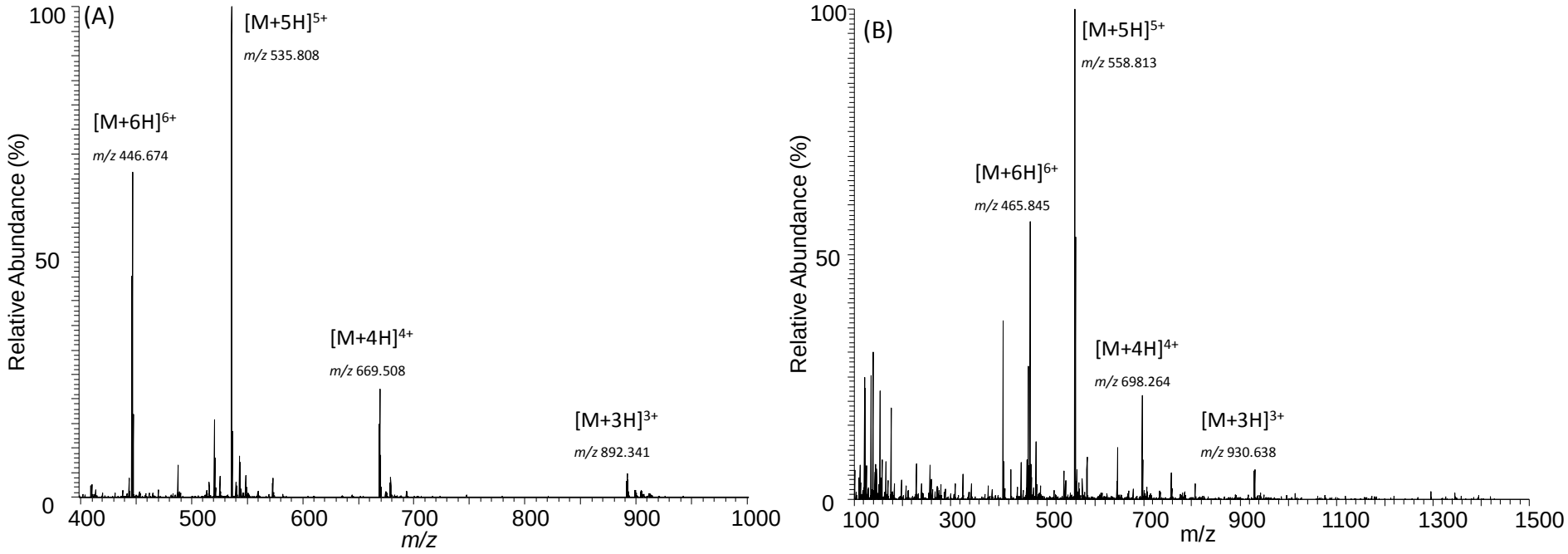


Figure 2-6. Mass spectra of (A) hepcidin-24, and (B) hepcidin-25, following direct infusion of separate 200 mg/L aqueous solutions containing 0.1 % (*v/v*) 3-NBA into the MS. Charge state and theoretical monoisotopic *m/z* are shown.

As hepcidin-25 gave the least intense signal of all hepcidins following infusion into the MS, and because the $[M+5H]^{5+}$ ion of hepcidin-25 was most intense, MS parameters were optimised using this ion, and the final MS conditions are presented in Chapter 2.2.2.

2.4 Optimising Liquid Chromatography conditions

LC columns investigated are listed in Table 2.2. For comparison of these columns, separate eluents consisting of 0.1 % (*v/v*) aqueous formic acid (eluent A), and 0.1 % (*v/v*) formic acid in acetonitrile (eluent B) were used with gradient elution. Starting condition; 5 % B, ramped to 100 % B over 10 minutes, held for 4 minutes then returned to initial conditions for 1 minute. Flow-rate was 0.40 mL/min, and column maintained at 60 ºC. Mass spectrometry conditions are as outlined above.

Table 2.2. Summary of LC columns investigated.

	Parameter				
Column	**Column length (mm)**	**Column diameter (mm)**	**Packing**	**Particle size (µm)**	**Pore size (Å)**
ACE	100	2.1	C18	2.0	100
ACE	100	2.1	C18	3.0	100
ACE	100	1.0	C18	3.0	100
ACE	100	2.1	Ultracore SuperC18	2.5	100
ACE	50	2.1	C18	3.0	100
Accucore	100	2.1	Phenyl hexyl	2.6	100
ProSwift	50	1.0	Phenyl	Monolithic column	

With regards to peak asymmetry, chromatographic resolution, and retention of analytes, the ACE C18 (100 x 1.0 mm i.d., 3.0 µm a.p.s) column was found to give optimum performance. Using this column, parameters such as flow-rate, column temperature, and the LC gradient were optimised.

The impact of changing eluent B from 100 % acetonitrile to 100 % methanol, and to a 1:1 mix of acetonitrile+methanol was also investigated. However, these additional solvents had a negligible effect on the chromatographic resolution of analytes, and rather increased the retention of all analytes on the LC column.

Final LC parameters are given in Table 2.3, and an extracted ion chromatogram is shown in Figure 2-7. There was a significant ‘dead volume’ in the LC system, calculated to be

approximately 0.35 mL; when taken into consideration it was calculated that hepcidins-20, -22, -24, and -25, eluted in approximately 19–30 % eluent B.

Table 2.3. Final LC parameters

Parameter	Condition
Eluents	A: 0.1 % (*v/v*) aqueous formic acid B: 0.1% (*v/v*) formic acid in acetonitrile
Column temperature	60 ºC
Flow-rate	0.1 mL/min during the 8 minute analysis time
Gradient	Starting condition 5 % B for 1 minute, ramped to 50 % B over 8 minutes, held for 1.5 minutes (100 % B, 0.4 mL/min) then immediately returned to initial conditions for 1.5 minutes (0.4 mL/min)
Injection volume	100 µL

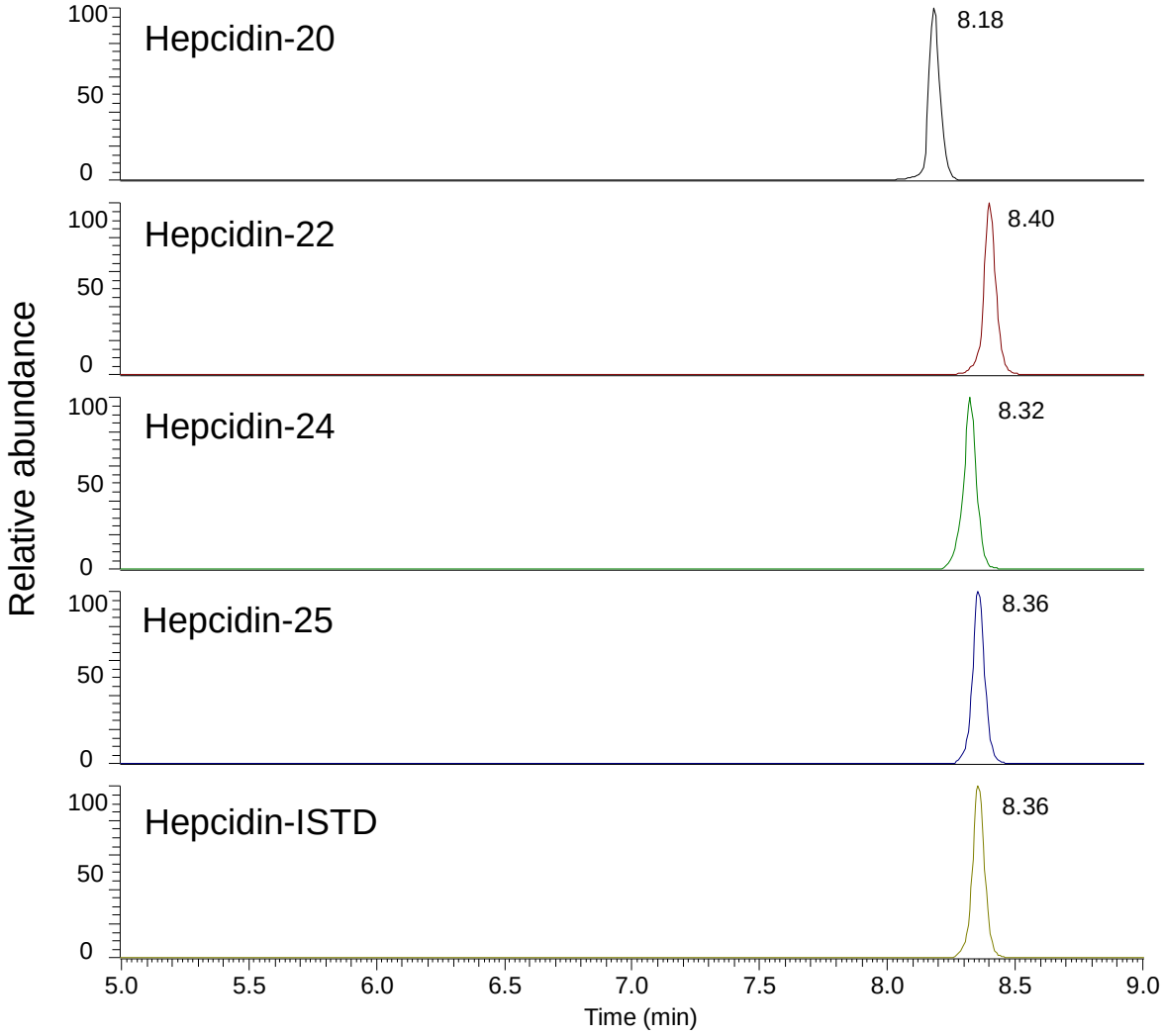


Figure 2-7. Extracted ion chromatograms for hepcidin-20, -22, -24, -25, and hepcidin-ISTD from the direct injection of an aqueous 100 µg/L solution.

2.5 Optimising Sample Preparation

Appropriate sample preparation is essential for any analyses undertaken by LC-MS in order to minimise ion suppression/enhancement from matrix components, and therefore improve accuracy and precision, as well as to minimise contamination of the LC and MS system. Reported techniques for the measurement of hepcidin-25 have included protein precipitation, solid-phase extraction, and functionalised magnetic nanoparticles. Recently, antibodies raised against hepcidin-25 bound to disposable automated research tips (D.A.R.Ts) have become available. Due to available resources within the laboratory only protein precipitation, solid-phase extraction and disposable automated research tips were investigated.

2.5.1 Protein precipitation

Protein precipitation was investigated using a number of different solutions, in order to identify which solution gave the best process efficiency. Sample (100 µL), and chilled protein precipitation solution (stored at 2–8 °C) were added (300 µL) to a 0.5 mL LoBind tube, and vortex mixed for 5 minutes, followed by centrifugation for 5 minutes (16,060 x *g*). Supernatant (100 µL) was diluted with deionised water (400 µL), and 100 µL analysed by LC-HR-MS. Results are given in Figure 2-8. All analytes (100 µg/L) were also added to portions of each precipitation solution and directly injected onto the LC-HR-MS (Figure 2-9) to give an indication of: (i) the solubility of the analyte in the solution, and (ii) the effect of the solution on the ionisation of each hepcidin.

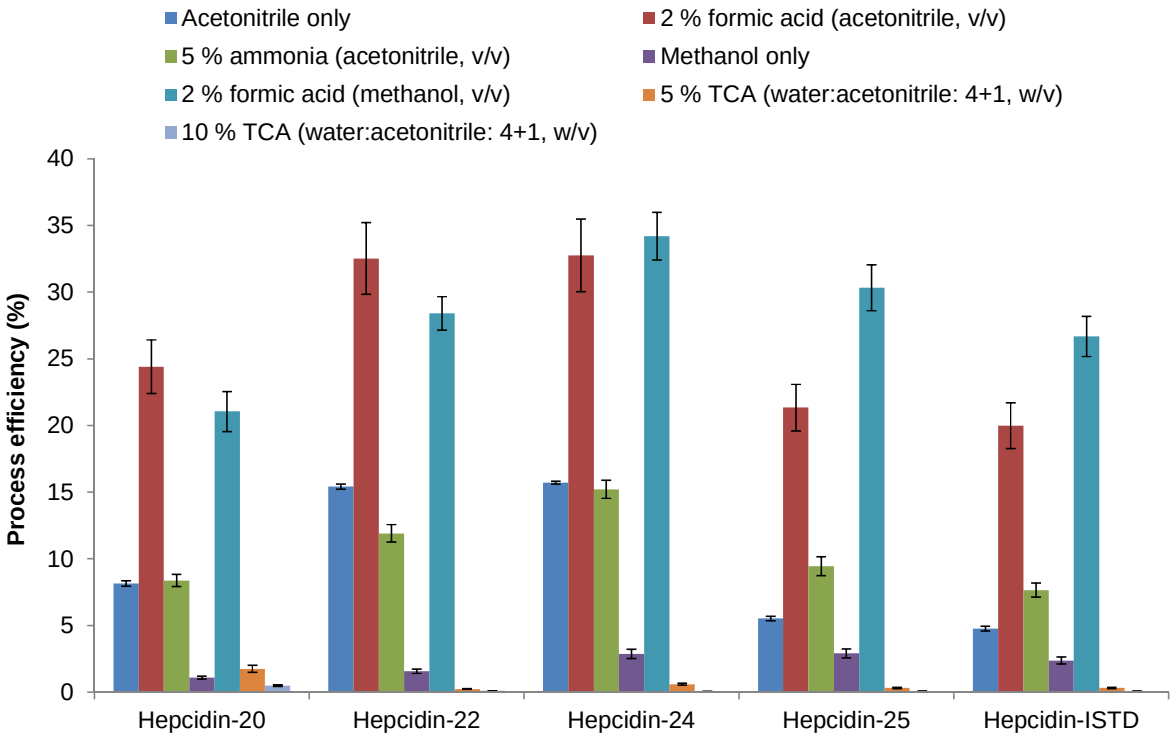


Figure 2-8. Process efficiency of all analytes from stripped human serum (100 µg/L) extracted using several protein precipitation solutions. Mean of triplicates, error bars represent ± standard deviation.

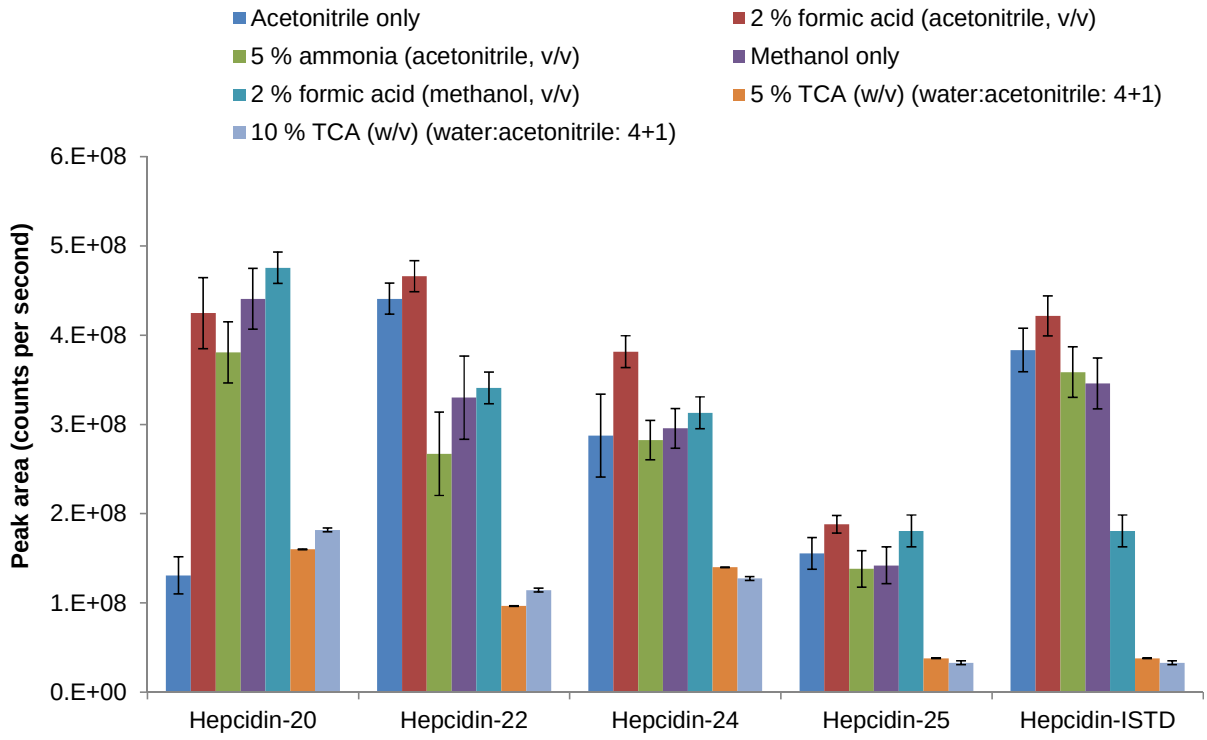


Figure 2-9. Peak areas of each hepcidin (100 µg/L) when added to individual portions of precipitation solution and directly injected onto the LC-HR-MS. Mean of triplicates, error bars represent ± standard deviation.

An acidified (2 % *v/v*) organic precipitation solution gave the best process efficiency, with little difference between acidified methanol and acetonitrile, although for hepcidin-25 the use of 2 % (*v/v*) formic acid in methanol gave a marginally better process efficiency, and therefore this was used in future experiments. The addition of 2 % formic acid reduced the pH of the supernatant from approximately pH 6 to below 1; the addition of both 5 and 10 % (*v/v*) trichloroacetic acid (TCA) also reduced the pH of the supernatant to below 1.

Comparing the results from Figure 2-8 and Figure 2-9, where the analytes were added to the precipitation solution and directly analysed, it is likely that acidification of the precipitation solution most likely displaces hepcidin from binding proteins during sample preparation. The poor process efficiency when using 5 and 10 % (*v/v*) TCA is likely due to protein bound analyte being precipitated (i.e. 'dragged down') together with the binding protein, combined with the poor solubility or ionisation, since the MS response is low without the presence of serum (Figure 2-9).

To ascertain process efficiency, matrix effects, and extraction recovery when using 2 % (*v/v*) formic acid in methanol as a protein precipitant, hepcidin-20, -22, and -25 were added to SHS (100 µg/L) and were calculated as described (Chapter 2.2.3). Due to a lack of hepcidin-24, and hepcidin-ISTD reference material at the time, these analytes were not investigated. Samples were prepared by addition of 2 % (*v/v*) formic acid in methanol (300 µL) to sample (100 µL) in a 0.5 mL LoBind tube, and vortex mixed for 5 minutes followed by centrifugation for 5 minutes (16,060 x g). Supernatant (100 µL) was diluted with deionised water (400 µL), and 100 µL analysed by LC-HR-MS. Results are given in Table 2.4.

Table 2.4 Extraction recovery, matrix effect, and process efficiency of hepcidin-20, -22, and -25 added to stripped human serum (100 µg/L), following precipitation with methanol containing 2 % (*v/v*) formic acid. Mean of triplicates. (Hepcidin-24, and hepcidin-ISTD could not be investigated due to a lack of reference material)

	Extraction recovery, % (SD)	Matrix effect, % (SD)	Process efficiency, % (SD)
Hepcidin-20	26.0 (0.9)	113.8 (1.8)	29.6 (1.5)
Hepcidin-22	25.5 (0.4)	121.4 (1.3)	31.0 (0.9)
Hepcidin-25	26.5 (0.8)	164.4 (0.1)	43.7 (1.3)

Extraction recovery was low for all analytes, and there were significant matrix effects for hepcidin-25. It was not possible to identify the origin of this matrix effect, a co-eluting compound was not identified in the mass spectrum, and alterations to the LC gradient did not show improvement.

Despite the presence of significant matrix effects, other parameters such as linearity and sensitivity were investigated. To ascertain these, hepcidin-20, -22, -24, and -25 were added to SHS (100 µg/L). This was serially diluted with SHS to produce further solutions of 1, 2, 5, 10, 20, and 50 µg/L. Samples were analysed in triplicate as described above, using 2 % (*v/v*) formic acid in methanol as the precipitation solution. Calibration curves for all analytes were linear (R^2 > 0.90). However, the lowest concentration that could be detected was 20 µg/L. This is not surprising given the considerable dilution of the sample that was performed first with the precipitating solution, and then with deionised water. Significant dilution of the supernatant with water is required to minimise the final concentration of methanol present in the supernatant to ensure that all analytes are retained on the LC column. To improve sensitivity, the effect of

evaporating the supernatant (in borosilicate tubs) to dryness under nitrogen, at ambient room temperature was ascertained. Process efficiency was poor using this approach with approximately 20 % of all analytes being recovered following evaporation and reconstitution. Dimethyl sulfoxide (DMSO) was added to the supernatant prior to evaporation in order to prevent complete evaporation of the extract and to keep the analytes in solution. However, addition of DMSO [20 % (*v/v*)] to the supernatant had a negligible effect on analyte recovery, after evaporation and reconstitution. Time spent vortex mixing (5–60 minutes), and the ratio of sample to precipitating solution (1+1, and 1+2) was investigated, but neither improved process efficiency or sensitivity.

Protein precipitation is a simple sample preparation technique that has been used for the measurement of hepcidin-25 prior to LC-MS/(MS) by several investigators (Murao *et al.,* 2007, Rochat *et al.,* 2013). However, given the considerable matrix effects present, the lack of sensitivity, and instability of all analytes upon evaporation, this technique was not considered suitable and not investigated any further.

2.5.2 Immunocapture

In immunocapture, pipette tips are embedded with a monolithic microcolumn activated with an antibody. Sample is aspirated using these tips in repeated binding cycles, and finally the captured analyte is eluted and analysed by LC-MS(/MS). The process of capturing the analyte and analysis by mass spectrometry is known as mass spectrometric immunoassay (MSIA). MSIA has already been used for the measurement of several clinically important proteins in human plasma and has been shown to be selective and highly sensitive (Krastins *et al.,* 2013). Recently, D.A.R.Ts bound with polyclonal, hepcidin-25 antibodies that have been raised against the C-terminus of hepcidin-25 (Figure 1-2) have become available. To assess the suitability of immunocapture, all hepcidins were added to deionised water (100 µg/L), and analysed according to the procedure in Table 2.5. Deionised water (65 µL) was added to the eluted sample to match the LC conditions at the time of injection, and 100 µL analysed by LC-HR-MS.

Table 2.5. Immunocapture procedure.

Step	Solution	Volume (µL)	Number of cycles	Cycle volume (µL)
1 - Wash	Deionised water	200	20	150
2 - Capture	Sample	700	500	250
3 - Wash	Phosphate buffered saline (PBS)	200	20	150
4 - Wash	Deionised water	200	20	150
5 - Elute	0.4 % (*v/v*) TFA (water: acetonitrile, 2+1)	100	100	75

Process efficiency ranged from 67–103 % (SD range: 11–16) for all analytes, clearly indicating that the antibody bound to the tip can capture all hepcidins in the absence of plasma or serum. However, when attempted using a SHS sample (500 µL), the tips often blocked and process efficiency was poor (< 1 % all analytes). To minimise tip blockage, and to displace hepcidins from plasma protein, sample (500 µL) was diluted with a range of aqueous diluents (750 µL, Table 2.6) into 1.5 mL LoBind tubes and vortex mixed for 60 minutes, followed by immunocapture. Process efficiency was unacceptable for all procedures (Table 2.6). In order to ascertain whether the low process efficiency obtained was due to matrix effects or poor extraction efficiency, extraction recovery, and matrix effects, were also ascertained following dilution of the sample (500 µL) with deionised water (750 µL) prior to immunocapture (Table 2.7).

Table 2.6. Process efficiency of all analytes (100 µg/L) from stripped human serum (500 µL), following dilution of the sample with several diluents prior to immunocapture. Mean of triplicates.

	Hepcidin-20		**Hepcidin-22**		**Hepcidin-24**		**Hepcidin-25**		**Hepcidin-ISTD**	
	PE (%)	SD	PE (%)	SD	PE (%)	SD	PE (%)	SD	PE (%)	SD
Deionised water	5.9	0.5	4.2	0.6	4.7	0.4	3.2	0.1	2.9	0.3
Deionised water and sonication	5.9	0.6	4.6	0.8	4.5	0.5	3.6	0.4	3.3	0.5
HBS-EP	1.6	0.1	2.2	0.3	2.3	0.2	1.2	0.4	1.2	0.3
Aqueous P20, 0.005 % (*v/v*)	4.9	0.4	2.7	0.3	4.0	0.4	2.4	0.4	2.4	0.2
Aqueous SDS, 0.3 % (*v/v*)	3.6	0.2	2.1	0.1	2.5	0.2	1.2	0.1	1.1	0.1
Aqueous Triton X-100, 0.3 % *v/v*	0.1	-	0.1	-	0.1	-	0.1	-	0.1	-

Table 2.7. Extraction yield, matrix effect, and process efficiency of all analytes (100 µg/L), following dilution of the sample with deionised water prior to immunocapture. Mean of triplicates.

	Extraction recovery, % (SD)	Matrix effect, % (SD)	Process efficiency, % (SD)
Hepcidin-20	5.6 (2.4)	95.0 (3.9)	5.9 (0.5)
Hepcidin-22	4.2 (1.5)	101.0 (1.7)	4.2 (0.6)
Hepcidin-24	4.5 (1.2)	96.0 (5.7)	4.7 (0.4)
Hepcidin-25	3.1 (7.1)	93.0 (10.1)	3.2 (0.1)
Hepcidin-ISTD	2.9 (5.1)	102.0 (7.1)	2.9 (0.3)

It is evident from the data in Table 2.7 that the poor process efficiency obtained when using immunocapture is due to low extraction recoveries as opposed to matrix effects. This may be due to unknown matrix components interfering with the binding of the analyte to the antibody, or because of significant analyte-protein binding, especially as the diluents used have a neutral pH, and therefore unlikely to displace protein bound hepcidins. Unfortunately, the use of diluents at extremes of pH (e.g. acidic) to disrupt protein binding is not feasible as this would denature the antibodies bound to the tips. Instead, to try and disrupt protein binding, protein precipitation with acetonitrile prior to immunocapture was undertaken. Acetonitrile (300 µL) was added to sample (100 µL), vortex mixed for 5 minutes and centrifuged for 5 minutes (16,060 x *g*). The supernatant was diluted with deionised water so that the acetonitrile content was < 5 % (*v/v*) prior to immunocapture. Process efficiency was still unacceptable (< 1 %, all analytes). Increasing the number of capture cycles from 500–1,500, and altering the ratio of sample to diluent did not improve process efficiency.

Even though immunocapture produces extremely clean extracts, and has been shown to be useful for measuring some peptides and proteins in human plasma (Krastins *et al.,* 2013), the extremely poor process efficiency of all hepcidins from SHS makes MSIA an unsuitable technique for the measurement of hepcidin-25 and *N*-truncated isoforms.

2.5.3 Solid Phase Extraction

SPE was investigated using Waters OASIS µElution plates. This is because: (i) these plates are available pre-prepared with a range of sorbents (Figure 2-10), (ii) allow the use of elution volumes as low as 25 µL, and (iii) are available in a 96-well plate format to aid throughput.

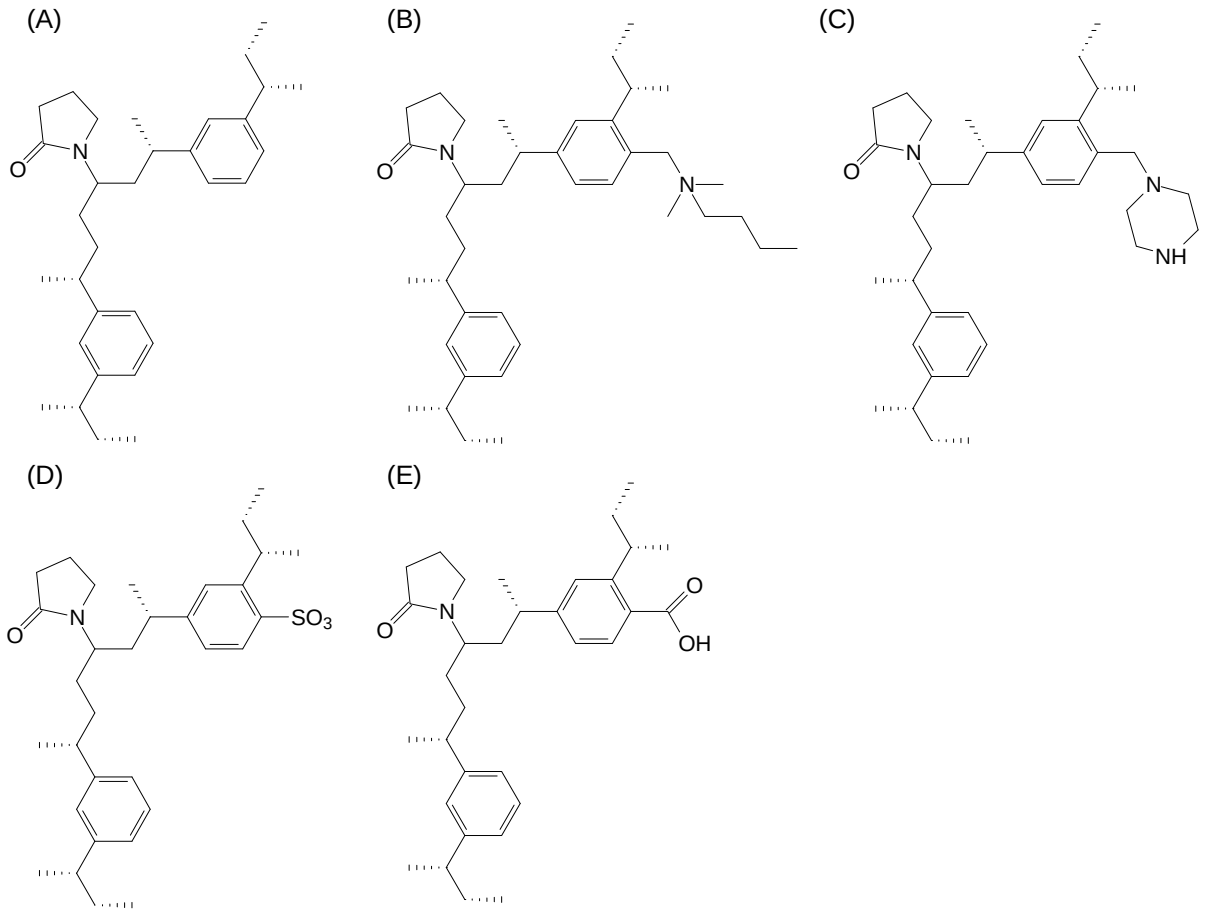


Figure 2-10. Surface-modification of the five SPE sorbents investigated; (A) hydrophilic lipophilic balanced (HLB); (B) maximum anion exchange (MAX); (C) weak anion exchange (WAX); (D) maximum cation exchange (MCX); and (E) weak cation exchange (WCX).

2.5.3.1 Sorbent comparison

The five sorbents shown in Figure 2-10, as well as the impact of loading and eluting the sample in an acidic, basic or neutral environment were investigated using a modified protocol available from Waters (Figure 2-11). Results are given in Figure 2-12, Figure 2-13, Figure 2-14, Figure 2-15, and Figure 2-16.

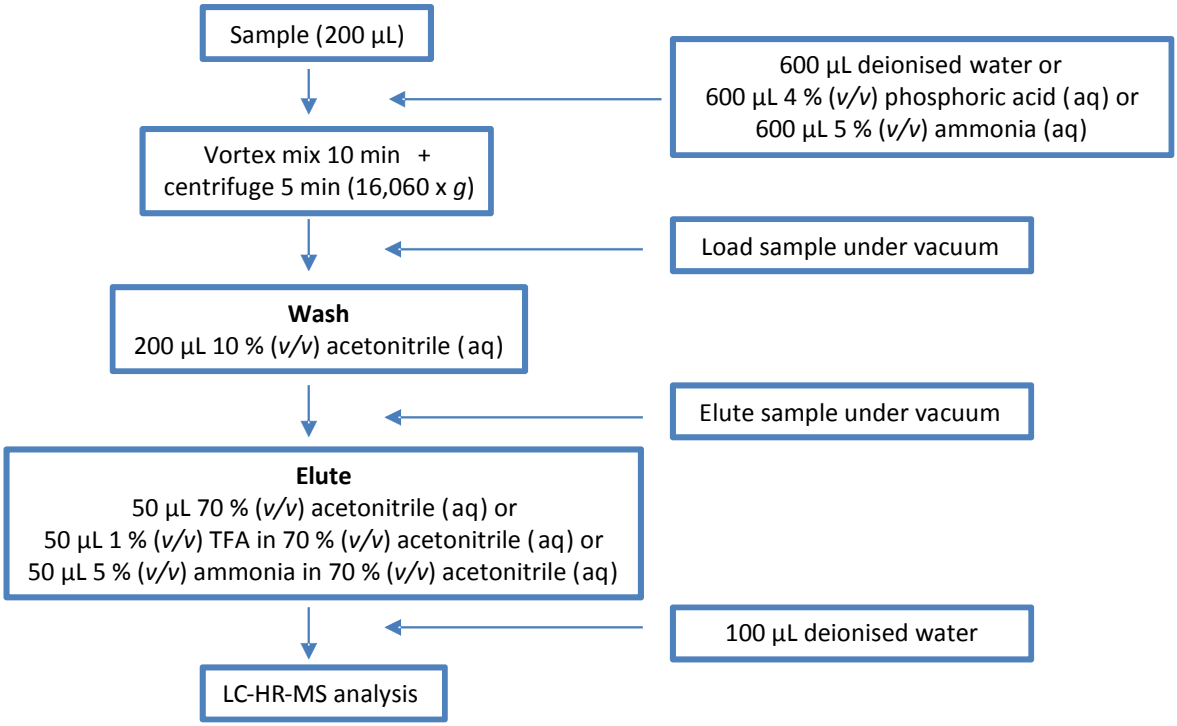


Figure 2-11. SPE procedure undertaken to compare 5 sorbents, and the effect of neutral, acidic, and basic loading and eluting conditions (prior to loading the sample, each well was conditioned with 200 µL of methanol and 200 µL of deionised water).

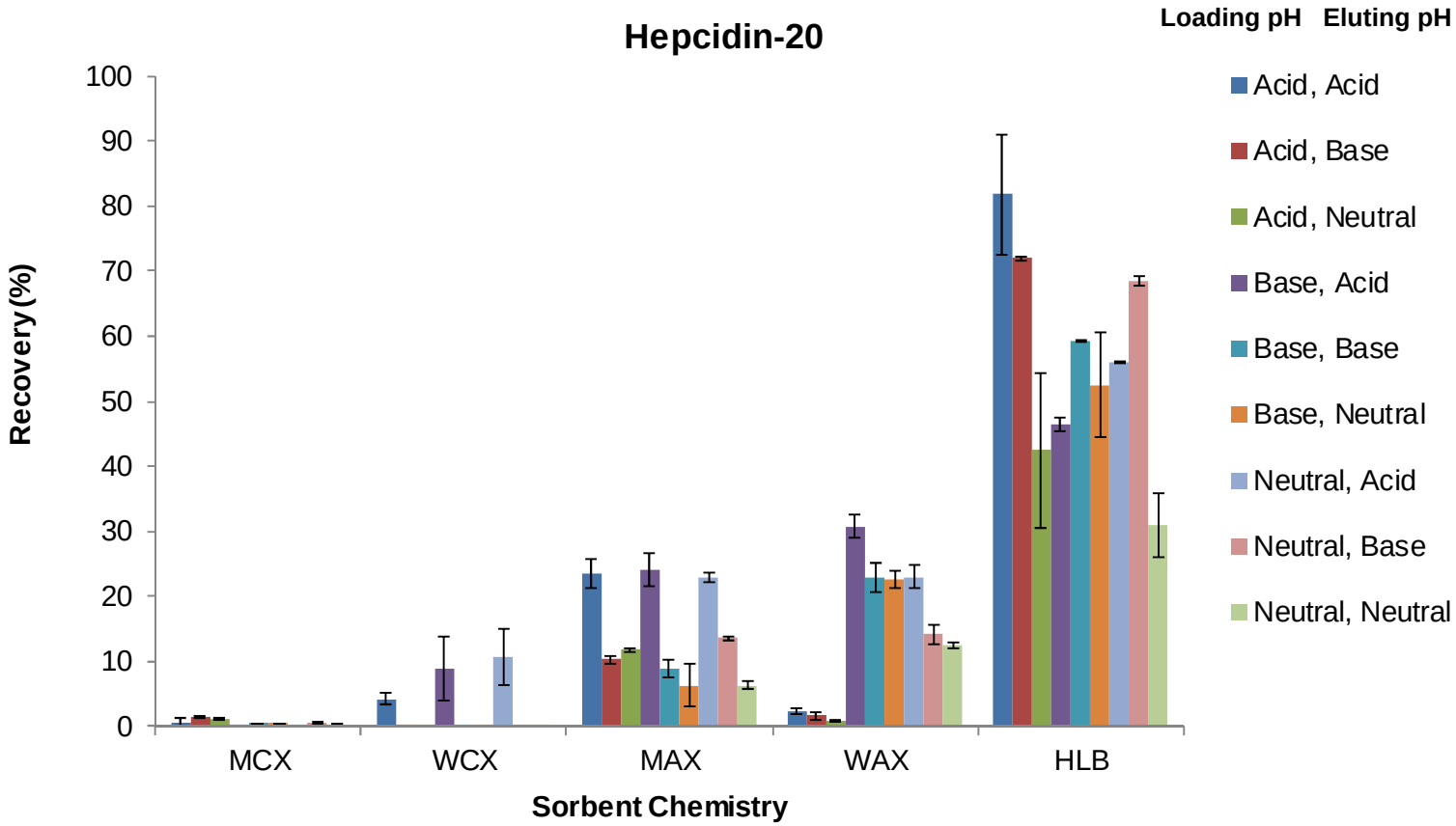


Figure 2-12. Process efficiency for hepcidin-20 following SPE using different sorbent chemistries and different loading and eluting conditions. Mean of triplicates, error bars represent ± standard deviation.

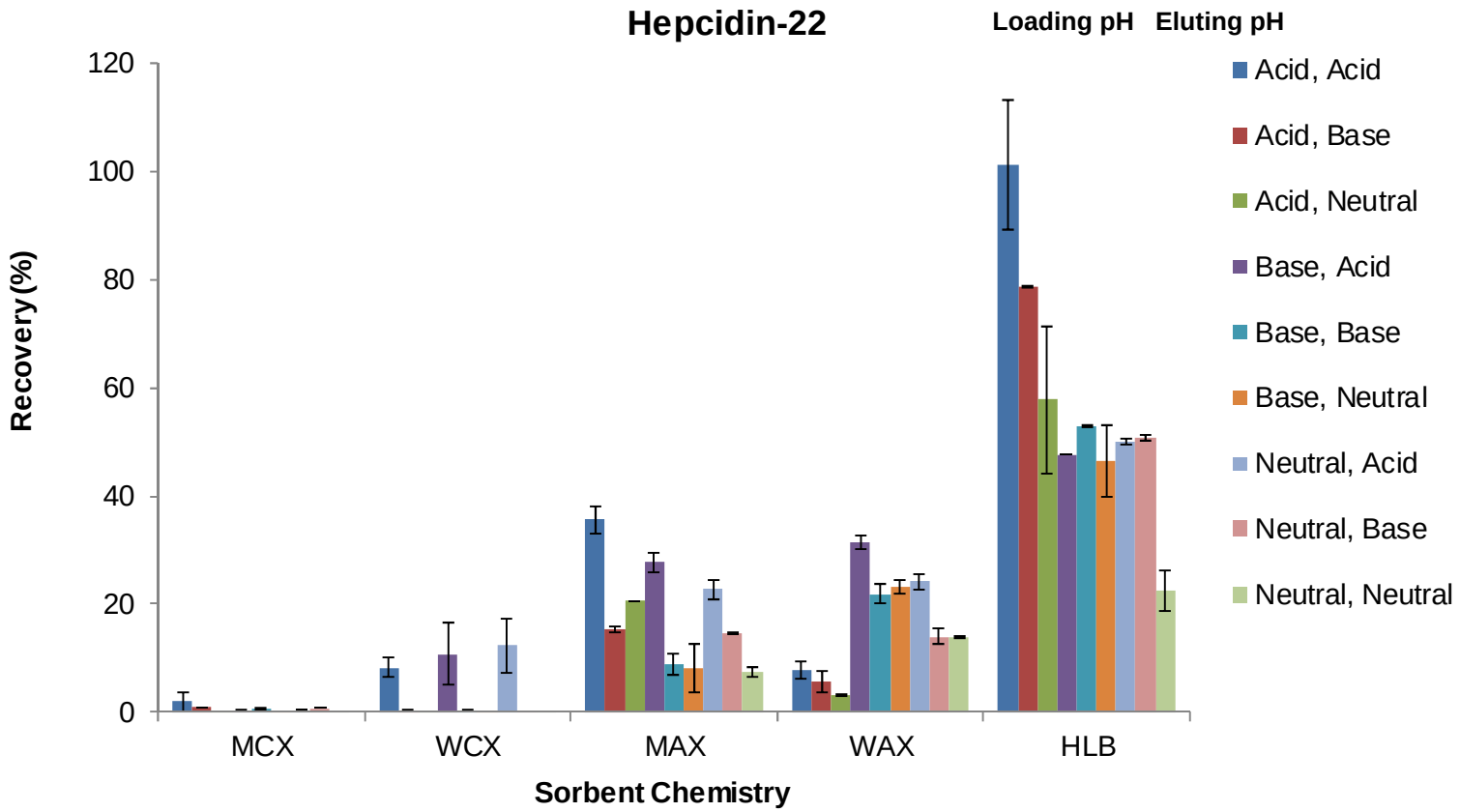


Figure 2-13 Process efficiency for hepcidin-22 following SPE using different sorbent chemistries and different loading and eluting conditions. Mean of triplicates, error bars represent ± standard deviation.

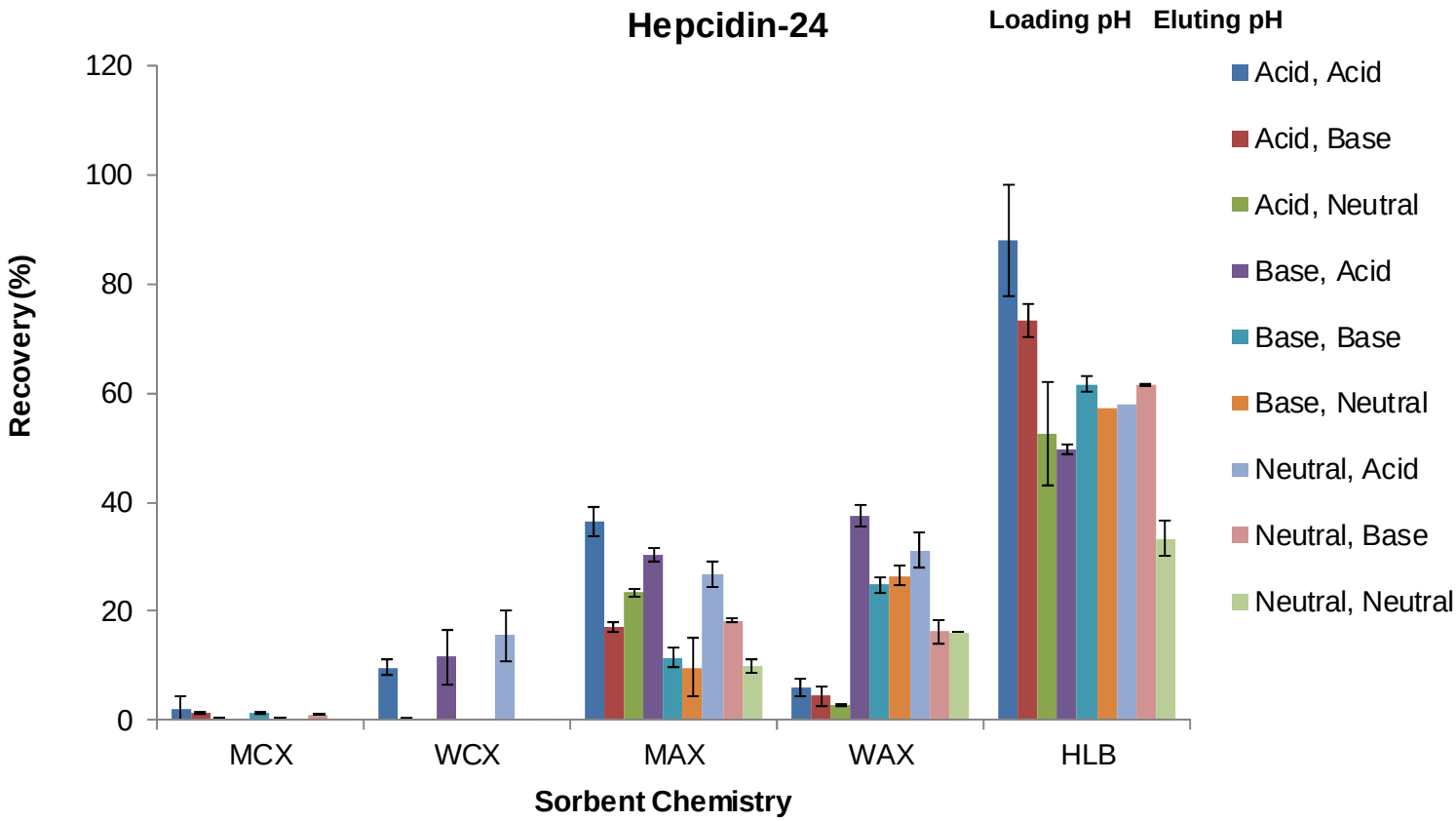


Figure 2-14 Process efficiency for hepcidin-24 following SPE using different sorbent chemistries and different loading and eluting conditions. Mean of triplicates, error bars represent ± standard deviation.

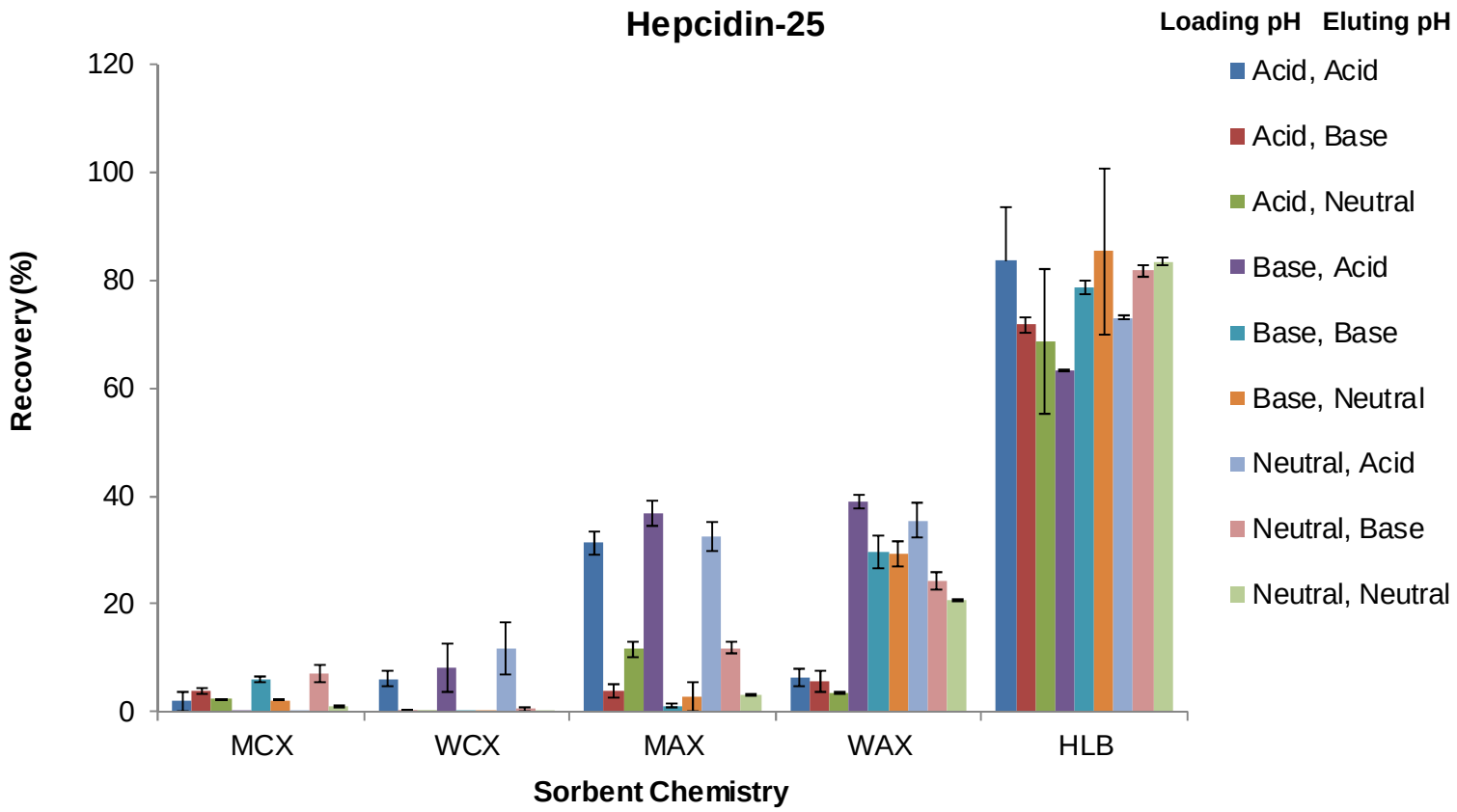


Figure 2-15 Process efficiency for hepcidin-25 following SPE using different sorbent chemistries and different loading and eluting conditions. Mean of triplicates, error bars represent ± standard deviation.

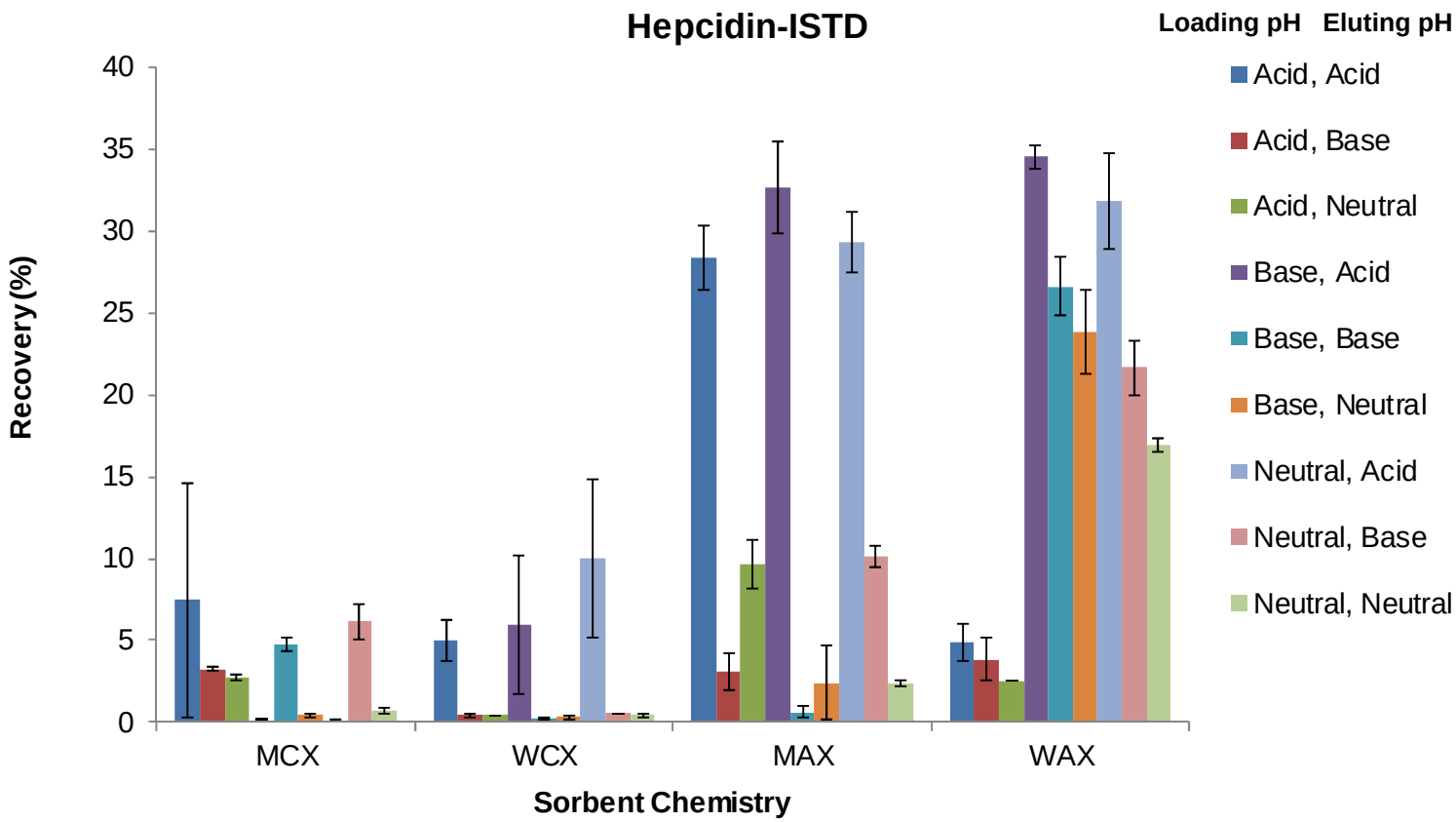


Figure 2-16 Process efficiency for hepcidin-ISTD following SPE using different sorbent chemistries and different loading and eluting conditions. Mean of triplicates, error bars represent ± standard deviation. Hepcidin-ISTD not investigated for HLB.

For hepcidin-20, -22, and -24, and to a lesser extent hepcidin-25, the HLB sorbent provided the greatest process efficiency compared to the other sorbent chemistries, especially when used with acidic loading and acidic eluting conditions, and was far superior to the process efficiency obtained when using protein precipitation or immunocapture.

To ascertain the cause of the low process efficiency for some of the sorbents, and to further compare all sorbents, the extraction recovery, matrix effects, and process efficiency were ascertained when the sample was loaded and eluted under acidic conditions. All analytes were also added to deionised water (100 µg/L) before extraction. Samples were prepared according to Figure 2-11, except that they were diluted with 4 % (*v/v*) aqueous phosphoric acid before loading, and eluted with 1 % (*v/v*) trifluoroacetic acid (TFA) in 70 % (*v/v*) aqueous acetonitrile. It was not possible to ascertain extraction recovery, matrix effects, and process efficiency using the WCX sorbent (all analytes), or the HLB sorbent for the hepcidin-ISTD due to limited resources. Results are given in Table 2.8. The HLB sorbent has the best process efficiency followed by the MAX sorbent. It is interesting to note that the process efficiency for all analytes in deionised water is far less than in SHS, this is likely due to poor solubility in a 100 % aqueous solution without the presence of soluble proteins, and analyte adsorption onto consumables.

Table 2.8. Extraction recovery, matrix effect, and process efficiency of hepcidins in SHS and deionised water at 100 µg/L following SPE. (HLB sorbent not investigated for hepcidin-ISTD due to limited resources) Mean of triplicates.

	Extraction recovery, % (SD)	Matrix effect, % (SD)	Process efficiency, % (SD) SHS	Process efficiency, % (SD) DEIONISED WATER
Hepcidin-20				
HLB	64 (3)	64 (1)	100 (10)	33 (3)
MCX	0 (0)	62 (15)	0 (0)	0 (0)
WAX	7 (2)	99 (3)	7 (1)	11 (1)
MAX	31 (3)	96 (5)	32 (4)	17 (6)
Hepcidin-22				
HLB	91 (12)	101 (0)	92 (19)	30 (4)
MCX	0 (0)	98 (24)	0 (0)	0 (0)
WAX	18 (5)	138 (8)	25 (3)	22 (2)
MAX	38 (2)	138 (1)	52 (6)	22 (7)
Hepcidin-24				
HLB	81 (2)	115 (1)	93 (21)	30 (4)
MCX	0 (0)	116 (27)	0 (0)	0 (0)
WAX	10 (2)	165 (5)	17 (3)	18 (3)
MAX	32 (3)	161 (5)	51 (8)	17 (6)
Hepcidin-25				
HLB	82 (10)	129 (0)	106 (24)	33 (4)
MCX	0 (0)	130 (31)	0 (0)	0 (0)
WAX	13 (4)	187 (4)	24 (4)	19 (3)
MAX	30 (4)	183 (4)	55 (8)	19 (7)
Hepcidin-ISTD				
MCX	1 (2)	135 (33)	1 (0)	0 (0)
WAX	14 (3)	187 (9)	26 (0)	24 (3)
MAX	35 (3)	182 (2)	63 (10)	31 (13)

As the use of a HLB sorbent with acidic loading and eluting conditions gave superior process efficiency compared to PPT and immunocapture, and because of other advantages of SPE (e.g. batch analysis on 96-well plates), SPE was further investigated and optimised as a suitable sample preparation technique.

2.5.3.2 Optimisation of sample and diluent volume

The impact of increasing sample volume on response was investigated by keeping the ratio of sample to 4 % (*v/v*) aqueous phosphoric acid constant (1+2), while the volume of sample was increased from 50–300 µL. The impact of increasing the volume of 4 % (*v/v*) aqueous phosphoric acid (100–400 µL) to a fixed volume of sample (100 µL), was also investigated using the procedure outlined in Figure 2-11.

When the volume of sample loaded onto the HLB sorbent increased so did the area of each analyte (Figure 2-17). However, when the volume of sample was doubled, the area increased by only 35–50 % as opposed to an expected 100 % increase. This may be due to the poor disruption of hepcidins from proteins. Increasing the volume of 4 % (*v/v*) aqueous phosphoric acid to sample did not have any significant effect on analyte area (Figure 2-18). These investigations indicated that the volume of sample loaded onto the HLB sorbent could be increased if improvements in sensitivity are required, and a minimum ratio of 1 part sample to 1 part diluent should be used.

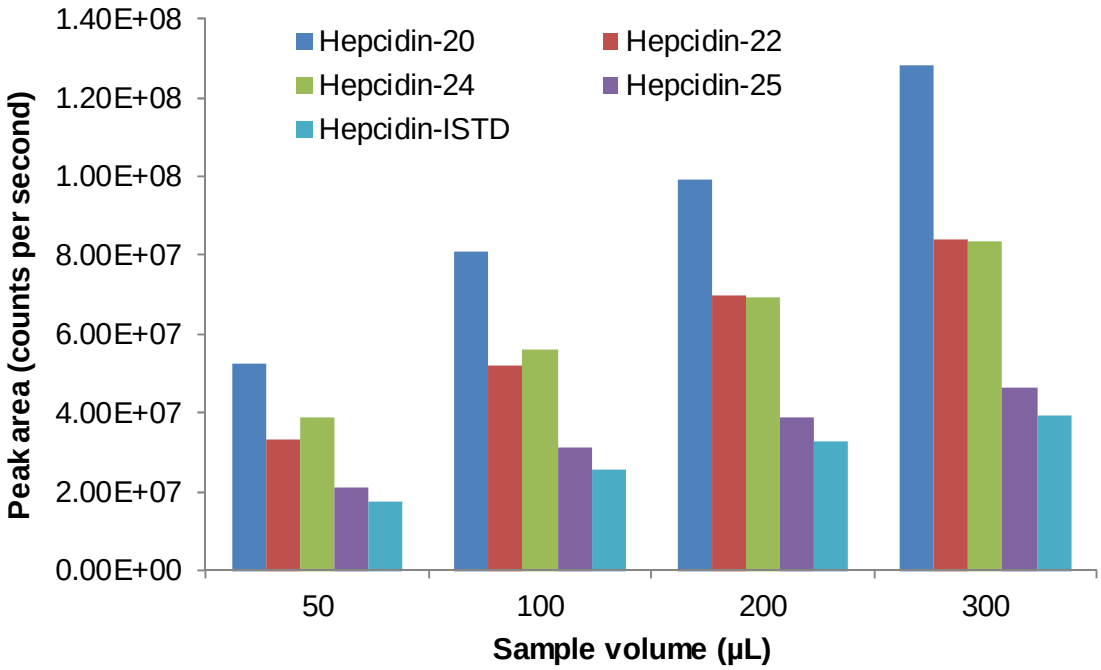


Figure 2-17. Effect of increasing the volume of sample (100 µg/L, all analytes) loaded onto the HLB sorbent, while keeping the ratio of sample to diluent (1+2) constant. Analysis undertaken in singlicate.

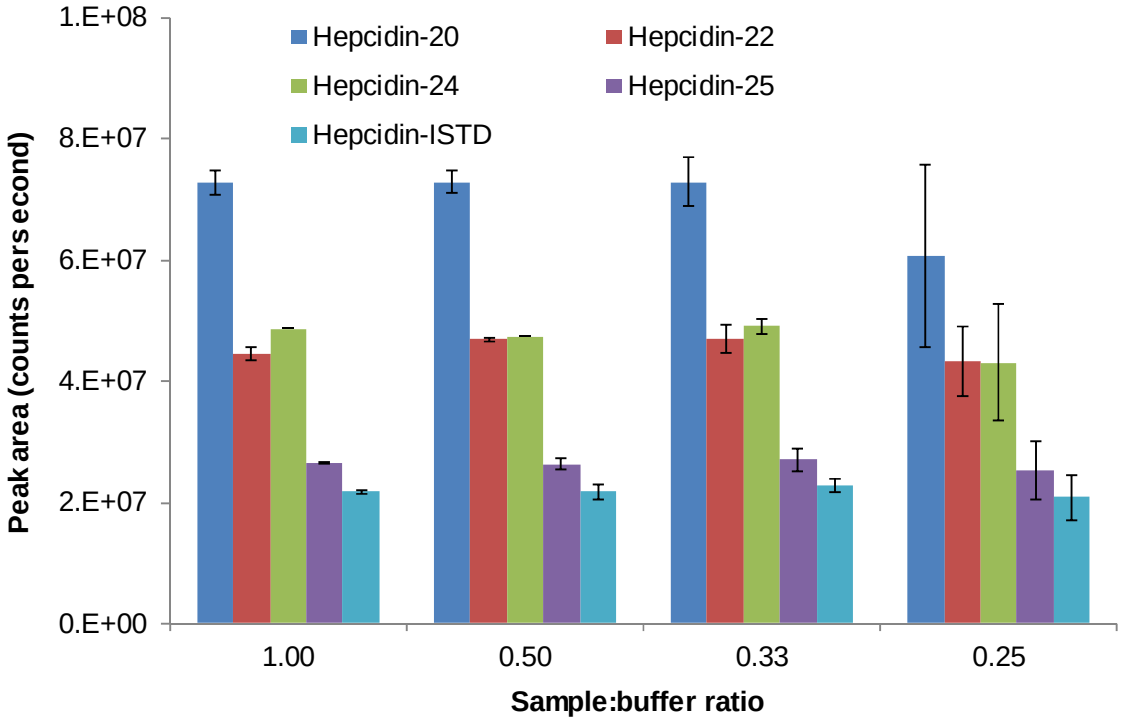


Figure 2-18. Effect of increasing the volume of sample relative to buffer (100 µg/L, all analytes) loaded onto HLB sorbent, while keeping the volume of sample (100 µL) constant. Mean of triplicates, error bars represent ± standard deviation.

2.5.3.3 Impact of sample preparation prior to loading

To assess which acid to dilute the sample with prior to loading onto the HLB sorbent, 4 % (*v/v*) aqueous phosphoric acid, and 0.1 % (*v/v*) aqueous formic acid were compared as diluents. Phosphoric acid and formic acid were selected, as when prepared with these final percentages they do not precipitate out proteins, but still reduced the pH of the supernatant to below 1. A solution of 10 % (w/v) aqueous TCA, containing 20 % acetonitrile was also investigated as a diluent. Sample (200 µL) was added to the appropriate diluent (600 µL) in a 1.5 mL LoBind tube, vortex mixed for 10 minutes before centrifuging (5 minutes, 16,060 x g) and loading all of the supernatant onto the HLB sorbent.

There was little difference in peak areas for all hepcidins between the use of 0.1 % (*v/v*) aqueous formic acid, and 4 % (*v/v*) aqueous phosphoric acid as diluents (Figure 2-19). However, precipitating out proteins using 10 % (*v/v*) aqueous TCA prior to loading onto the SPE sorbent gave considerably lower peak areas, consistent with the findings from Section 2.5.1.

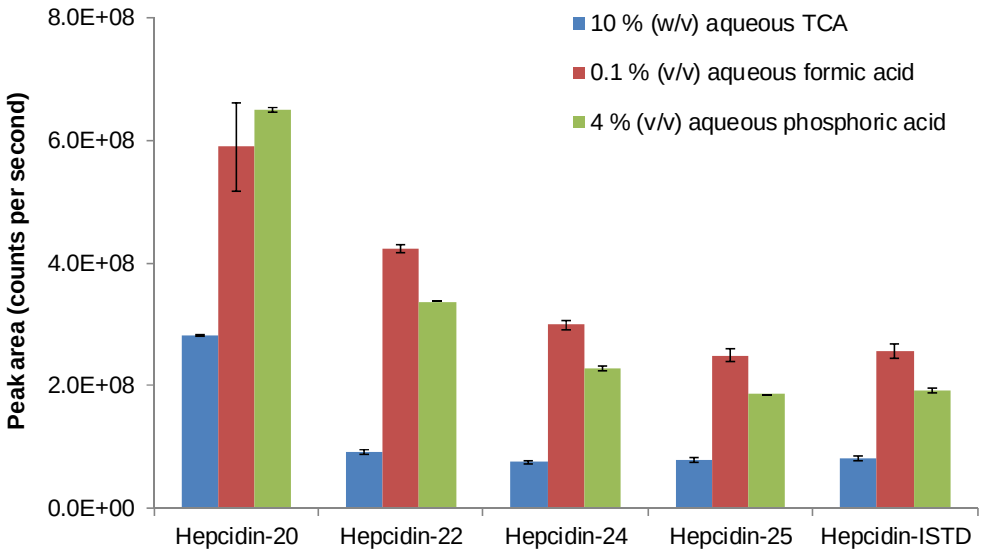


Figure 2-19. Comparison of different diluents on peak areas. Mean of triplicates, error bars represent ± standard deviation.

Although there was little difference between the use of phosphoric and formic acid, aqueous formic acid (0.1 %, *v/v*) as a diluent was marginally better for hepcidin-25, and is consistent with other investigators who have used this approach prior to loading onto the SPE sorbent (Li *et al.,* 2009; Wolff *et al.,* 2013; Lefebvre *et al.,* 2015). Increasing the percentage of formic acid from 0.1–1.0 % (*v/v*) did not improve response, therefore a 0.1 % (*v/v*) aqueous formic acid solution was used to minimise protein precipitation and therefore reduce the chance of the analyte being 'dragged down' by binding proteins.

2.5.3.4 Optimisation of wash solution

Wash solutions consisting of either 20 % (*v/v*) aqueous acetonitrile alone, 20 % (*v/v*) aqueous methanol alone, or either with 4 % (*v/v*) phosphoric acid or 5 % (*v/v*) ammonia added were compared. There was little difference in the area of all analytes (Figure 2-20) between wash solutions. However, a wash solution containing 20 % (*v/v*) aqueous acetonitrile alone gave a slightly better response for hepcidin-25.

The use of acetonitrile in the wash solution was further optimised by comparing deionised water containing varying percentages (*v/v*) of acetonitrile (0–50 %) either alone, with 4 % (*v/v*) phosphoric acid, or 5 % (*v/v*) ammonia (Figure 2-21). The optimum percentage of acetonitrile in the wash solution was considered to be 20 % (*v/v*), without the addition of phosphoric acid or ammonia.

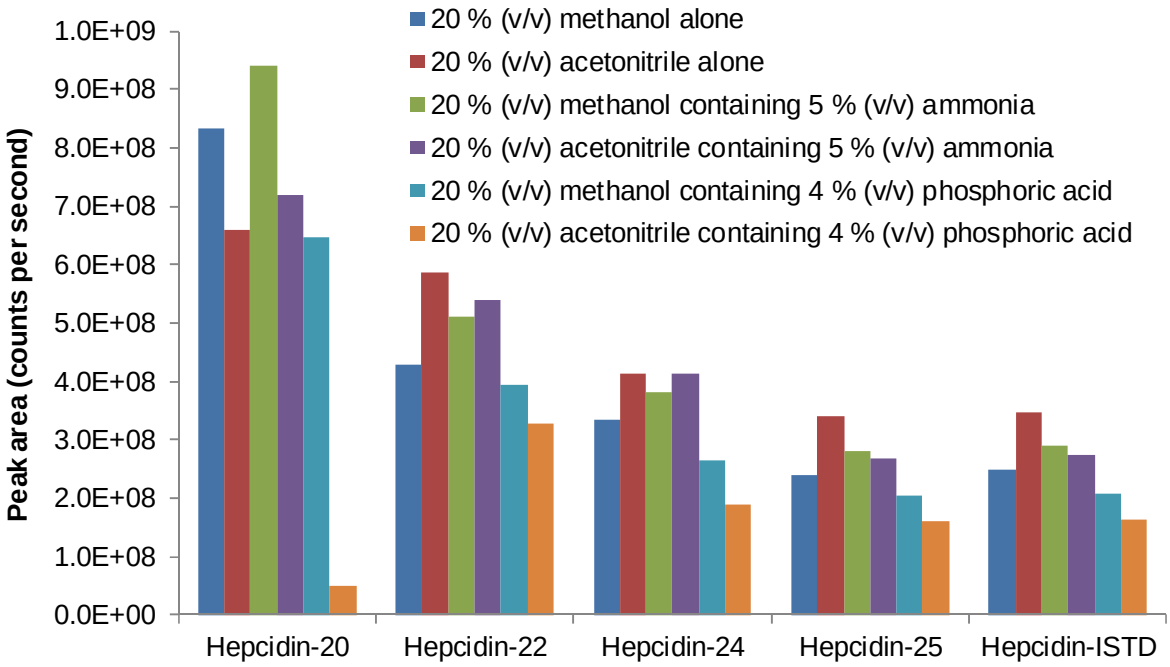


Figure 2-20. Effect of different wash solutions on analyte peak areas. Analysis undertaken in singlicate.

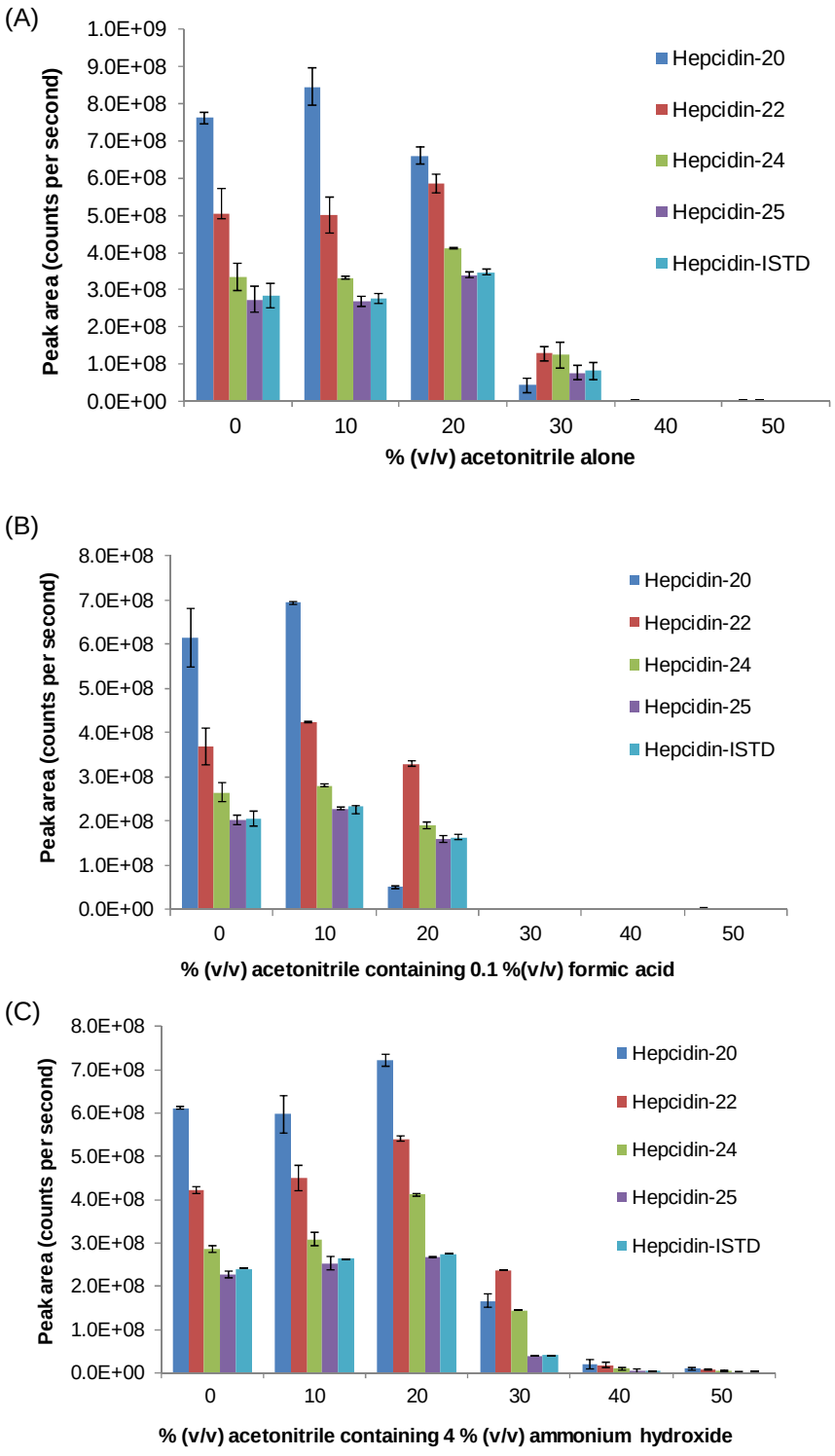


Figure 2-21. Effect of varying concentrations of; (A) aqueous acetonitrile alone, (B) aqueous acetonitrile with 0.1 % (*v/v*) formic aid, and (C) aqueous acetonitrile containing 4 % (*v/v*) ammonium hydroxide. Mean of triplicates, error bars represent ± standard deviation.

2.5.3.5 Optimisation of eluting solution

The percentage of acetonitrile in the eluting solution was optimised over the range 30–90 % (*v/v*) while keeping the elution volume constant (50 µL). The elution volume was also investigated over the range 20–80 µL, while keeping the acetonitrile content constant (70 %, *v/v*). The optimum percentage of acetonitrile in the eluting solution was considered to be 60 %, and the optimum elution volume was 70 µL (Figure 2-22).

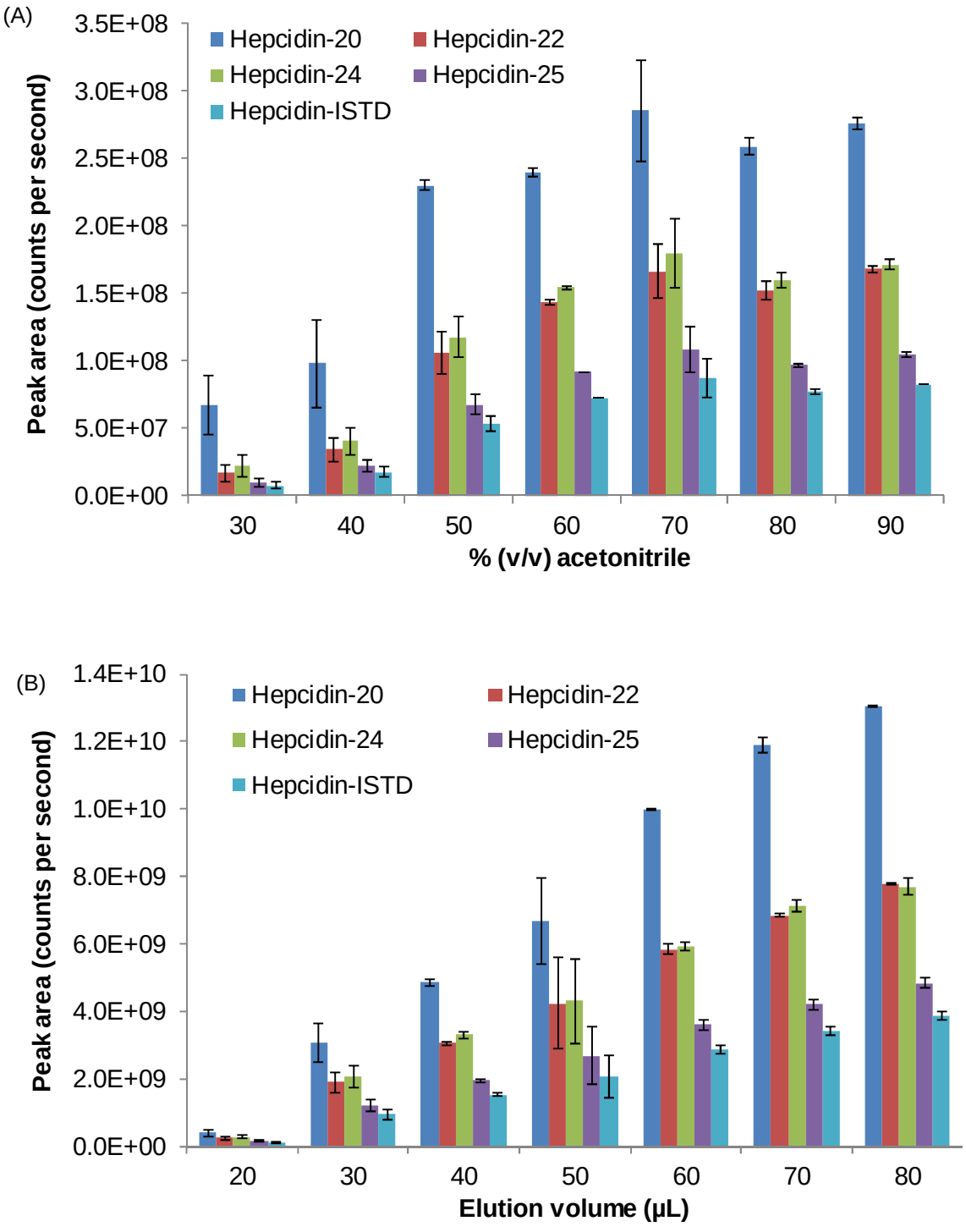


Figure 2-22. Effect of; (A) increasing the percentage of acetonitrile (*v/v*) in a constant elution volume (70 µL), and; (B) increasing the volume of elution solution, while keeping the percentage of (*v/v*) acetonitrile constant (70 %). Mean of triplicates, error bars represent ± standard deviation.

2.5.3.6 Final SPE method

Following much optimisation, the final SPE method was as follows:

- Dilute sample (200 µL) with 0.1 % (v/v) aqueous formic acid (600 µL)
- Vortex mix for 5 minutes, followed by centrifugation for 5 minutes (16,060 x g)
- Load supernatant onto HLB sorbent under vacuum
- Wash with 20 % (*v/v*) aqueous acetonitrile (200 µL)
- Elute with 60 % (*v/v*) aqueous acetonitrile containing 1 % (*v/v*) TFA (70 µL)
- Add 100 µL of deionised water to extract
- Inject 100 µL onto LC-HR-MS

Using the above method, a calibration curve over the range 1–200 µg/L (all analytes) was prepared in SHS, and extracted per the method described. The calibration was linear for all analytes ($R^2 > 0.90$). Therefore, this method was considered suitable for the measurement of all hepcidins, and this optimised method was then ready for validation.

2.6 Discussion

Published mass spectrometry based methods for the measurement of hepcidin-25 broadly fall into 2 groups (i) SELDI- and MALDI-TOF-MS based, and (ii) LC-MS/(MS) based. No published method quantifies all commercially available isoforms of hepcidin-25 (i.e. hepcidin-20, -22, -24, and -25). SELDI- and MALDI-TOF-MS are widely used in research environments for qualitative analysis. When used for quantitative analysis, advantages include high-throughput of samples; some disadvantages, however, include poor reproducibility between samples in-part due to matrix effects, and relatively low mass resolution (Szájli *et al.,* 2008). The use of a suitable internal standard can help to improve the precision of measurements, and if the assay has been thoroughly developed and evaluated SELDI and MALDI can be applicable to the measurement of hepcidin-25. Several such assays have been published (Ward *et al.,* 2008; Laarakkers *et al.,* 2013). As SELDI/MALDI instruments are mainly located within research environments it is not surprising that the first published methods for hepcidin-25 quantitation used these techniques. However, published hepcidin-25 methods, have increasingly used LC-MS/MS, in-part because

this technique is widely used for quantitative analysis and that these instruments are more widely found in clinical chemistry laboratories. However, LC-HR-MS is becoming increasingly popular in laboratories for the quantitative analysis of peptides/proteins and small molecules.

The results from proficiency testing schemes can be useful to compare techniques, and this approach has been undertaken for vitamin D (Couchman *et al.,* 2012). Unfortunately, no such scheme exists for hepcidin-25, and even though there have been several round-robins undertaken (Kroot *et al*., 2009, Kroot *et al*., 2012) participant's assays are grouped into either immunochemical or mass spectrometric, and no further detail is given in-order to provide anonymity of each participant's results. Therefore, the performance of different mass-spectrometric techniques (i.e. MALDI vs SELDI, or vs LC-MS/MS) cannot be compared, and even if this information was available there would be too few assays per group to allow meaningful interpretation.

With regards to sample preparation published LC-MS based methods for hepcidin-25 have used either protein precipitation (Murao *et al.,* 2007; Rochat *et al.,* 2013), ultrafiltration (Kobold *et al.,* 2008), magnetic nanoparticles (Bansal *et al.,* 2010) or solid phase extraction (Murphy *et al.,* 2007; Wolff *et al.,* 2013; Li *et al.,* 2009; Li *et al.,* 2015; Lefebvre *et al.,* 2015). In the work undertaken here however, protein precipitation using an organic solvent (e.g. acetonitrile) or aqueous TCA, prior to analysis by LC-MS gave unacceptable process efficiencies and matrix effects. Instead, Waters Oasis µElution SPE plates were used as they are available in 96-well formats readily applicable to high-throughput analyses, come in a range of sorbent chemistries, and allow concentration of the analyte through the use of small elution volumes. The HLB sorbent provided optimum process efficiency with minimal matrix effects, as compared to other ion-exchange sorbents. This is in keeping with several other reported methods that have used Waters Oasis µElution SPE plates with the HLB sorbent (Murphy *et al.,* 2007; Li *et al.,* 2009; Li *et al.,* 2015; Wolff *et al.,* 2013). A recently published method (Lefebvre *et al.,* 2015) used Waters OASIS MAX µElution plates after mixing the sample with 0.1 % (*v/v*) aqueous formic acid, and eluting with 5 % (*v/v*) formic acid in 72.5 % (*v/v*) aqueous acetonitrile. However, this approach is counterintuitive as all hepcidins have an isoelectric point of 8.22–8.53, and when loaded onto a MAX sorbent in an acidic environment the mechanism of retention would not be ion-exchange but rather reversed-phase. Furthermore, all hepcidins have

only 18 to 25 % of their amino acids ionisable, of which most are situated next to disulfide bridges and would be unlikely to interact with ion-exchange functions present on the sorbents.

Despite immunocapture having been used a number of other clinically relevant proteins (Krastins *et al.,* 2013); it was not suitable as a sample preparation technique for hepcidin primarily due to poor process efficiency. It is not clear why process efficiency was low when using this approach, but it is likely that other unknown endogenous compounds interfered with antibody binding.

With regards to chromatography, even though all hepcidins had similar retention times they were almost resolved at baseline, and the use of a microbore LC column (i.e. internal diameter 1 mm) allowed the use of a low flow-rate (0.1 mL/min) without an excessively long analysis time. Since ESI is concentration-dependent, a low flow-rate will not cause a reduction in sensitivity, but rather improve sensitivity as ionisation of the analyte is much improved at low flow flow-rates.

Traditionally, triple quadrupole mass–analysers have been used for the quantitation of small molecules, including peptides and proteins. This is in part, due to the selectivity and sensitivity that can be obtained with these instruments. However, hepcidin-25 can absorb substantial fragmentation energies before fragmentation occurs, and when hepcidin-25 does fragment, there are many product ions of low or variable intensities (Bansal *et al.,* 2009). Those methods that have used this approach, monitor product ions which are either of low abundance (Murphy *et al.,* 2007) or of a low *m/z* (Murao *et al.,* 2007), which lack specificity. Furthermore, these methods monitor only a single charge state, and a single isotope of that charge state, however there is the possibility that charge state distribution may differ from sample to sample. The method developed here, has the advantage that the top 5 most abundant isotopes (88 % of the theoretical charge) of the top 3 most predominant charge states are monitored and used for post-acquisition data processing, thereby ensuring that any charge state or isotope variability between samples is accounted for. By using high resolution-mass spectrometry, the method here resolves almost all precursor ions entering the mass spectrometer, which improves selectivity and aids accurate quantitation, especially when used with an appropriate mass extraction window. Also, by using high resolution-mass spectrometry and acquiring data in full scan mode across a broad mass range, post-acquisition interrogation of data for other isoforms

of hepcidin (i.e. hepcidin-19, -21, -23) is possible, as is presented in Chapter 3, when investigating the stability of hepcidin isoforms.

2.7 Conclusions

The proposed method allows the quantitation of all available hepcidin isoforms in a single analysis. By acquiring data in full scan mode the top 5 isotopes of each charge state to be summed, ensuring that most of the analyte charge can be used in post-acquisition data processing. Despite the use of protein precipitation having been previously used for the measurement of hepcidin-25, neither this technique, nor immunocapture, was suitable for the work undertaken here due to poor process efficiencies. Solid Phase Extraction, although time-consuming to optimise, gave superior process efficiency, and the use of 96-well plates has the advantage that high-throughput analyses can be undertaken.

Chapter 3 Method Validation

3.1 Introduction

Method validation is a necessary process to demonstrate that an analytical method is suitable for its intended use. Parameters such as accuracy, precision, and ion suppression (if MS is being used) should be assessed, as should stability of the analyte in prepared extracts and in biological samples. Comparability between matrix types (i.e. plasma vs serum) should also be ascertained if the sample type may vary, and where possible the developed method should be compared against other appropriately validated methodologies. As significant variability between assays for hepcidin-25 has been reported (Kroot *et al.,* 2009; Kroot *et al.,* 2012; van der Vorm *et al.,* 2016), a method-based reference range in healthy individuals should also be established.

The aims of this chapter are as follows:

- Validate the developed LC-HR-MS assay for serum/plasma hepcidin-25 and *N*-truncated isoforms
- Compare hepcidin isoform concentrations in paired human plasma and serum samples
- Investigate the stability of all hepcidin isoforms when added to stripped-human serum and in clinical samples
- Measure all hepcidin isoforms in samples from healthy volunteers to create a method-based reference range

3.2 Materials and methods

All chemicals, reagents and consumables used are given in Section 2.2.

3.2.1 Preparation of calibration and internal quality control solutions

Stock solutions (100 mg/L, corrected for purity where required) of each individual hepcidin, including internal standard, were prepared in 30 % (*v/v*) aqueous acetonitrile containing 0.1 % (*v/v*) formic acid. Stripped human serum was analysed before use to prepare calibration and IQC solutions, and no analytes were detected (LLoQ 1 µg/L). Two calibration working solutions (1 mg/L and 4 mg/L, all analytes) were prepared by appropriate dilution of individual stock solutions with analyte-free stripped human serum into grade A volumetric glassware. A separate internal quality control (IQC) working solution was prepared (4 mg/L, all analytes) in analyte-free stripped human serum.

To prepare calibration and IQC solutions (Table 3.1), appropriate volumes of working solutions were pipetted into grade A volumetric glassware and made up to volume with analyte-free stripped human serum. After standing overnight (2–8 ºC) and after thorough mixing, portions of all solutions were transferred to 0.5 mL LoBind tubes and stored at -20 ºC until required.

Working internal standard (IS) solution (1 mg/L) was prepared by dilution of the hepcidin-ISTD stock solution with 30 % (*v/v*) aqueous acetonitrile containing 0.1 % (*v/v*) formic acid, and portions stored at -20 °C in 0.5 mL LoBind tubes until required.

Table 3.1 Nominal analyte concentrations: stripped human serum calibration and IQC solutions.

Analyte	Calibration solution concentration (µg/L)							IQC solution concentration (µg/L)		
	1	2	3	4	5	6	7	A	B	C
Hepcidin-20	1	2	5	10	25	50	100	4	8	25
Hepcidin-22	1	2	5	10	25	50	100	4	8	25
Hepcidin-24	1	2	5	10	25	50	100	4	8	25
Hepcidin-25	1	2	5	10	50	100	200	8	75	150

3.2.2 Instrumentation and conditions

An Aria Transcend LX-II system (ThermoFisher Scientific, San Jose, USA) consisting of four Accela 600 high-pressure quaternary pumps, valve interface module, column oven and CTC PAL autosampler was used with a Q-Exactive mass spectrometer (ThermoFisher Scientific). Liquid chromatography and mass spectrometer instrument control was performed using Aria OS (version 2.0, ThermoFisher Scientific), and Xcalibur software (version 2.2, ThermoFisher Scientific), respectively. System eluents were: (A) 0.1% (*v/v*) aqueous formic acid, and (B) 0.1% (*v/v*) formic acid in acetonitrile. The total eluent flow rate was delivered at 0.1 mL/min, using the following step wise gradient; 5 % B for 1 minute, ramped to 50 % B over 8 minutes, then stepped to 100 % for 1.5 minutes (flow-rate 0.4 mL/min) before returning to starting conditions for 1.5 minutes (flow-rate 0.4 mL/min). Prepared samples (100 µL) were injected onto an ACE C18 modified silica column (100 x 1.0 mm i.d, 3 µm a.p.s) maintained at 60 ºC. MS detection was carried out in positive mode using heated electrospray ionization [spray voltage 4.0 kV; temperatures: vaporizer 150 ºC; capillary 350 ºC; auxiliary, sheath and sweep gases 10, 35 and 0 (AU), respectively, S-lens voltage 90 V]. The total analysis time was 12 minutes. Full-scan MS data were acquired using a resolution setting of 70,000, defined as full width at half maximum at *m/z* 200, with a scan range of 400–1000 *m/z* (Orbitrap settings: maximum injection time 200 ms, automatic gain control [AGC] target 3 x 10^6 ions). Post-acquisition data processing used Xcalibur software, and peak areas were generated by filtering full scan data using a mass extraction window of ± 10 ppm based on theoretical monoisotopic *m/z* values of the five most abundant isotopes of the $[M+3]^{3+}$, $[M+4]^{4+}$, and $[M+5]^{5+}$ charge states (Table 2.1).

Peak area ratios (hepcidin-20, -22, -24, and -25 to hepcidin-ISTD) were measured and used to construct calibration curves ($1/x^2$ weighting, not forced through zero). Qualitative analyte identification criteria were: (i) LC retention time within 10 s of that initially ascertained (including co-elution with the internal standard for hepcidin-25), and (ii) *m/z* value of the protonated pre-cursor ion within ± 10 ppm. Samples where the analyte concentration measured was above the top calibrator were diluted appropriately with analyte-free stripped human serum prior to reanalysis.

3.2.3 Sample preparation

Sample (200 µL) was added to a 1.5 mL LoBind tube together with IS working solution (10 µL, 1,000 mg/L), and 0.1 % (*v/v*) aqueous formic acid (600 µL), followed by vortex mixing for 5 minutes, and centrifugation for 5 minutes (16,060 x *g*). An Oasis HLB µElution, 96-well, SPE plate was conditioned by addition of methanol (200 µL) followed by deionised water (200 µL) while under vacuum. The entire supernatant was loaded onto the conditioned 96-well SPE plate, and allowed to flow freely under gravity for 5 minutes after which vacuum was applied until no supernatant remained. The plate was washed sequentially with 20 % (*v/v*) aqueous acetonitrile (200 µL), and then deionised water (200 µL), both under vacuum. Analytes were eluted with 70 % (*v/v*) aqueous acetonitrile containing 1 % (*v/v*) TFA under vacuum (60 µL). After elution, 0.1 % (*v/v*) formic acid (70 µL) was added to each extract. The 96-well plate was then transferred to the autosampler, and 100 µL injected onto the LC-HR-MS.

3.2.4 Method validation

3.2.4.1 Mass accuracy

To assess mass accuracy, mass error (*m/z Δ*) was calculated for each individual isotope (top 5) of the $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5}$ ions of each calibration solution within one batch (intra-assay) and between batches (inter-assay, N = 5).

3.2.4.2 Intra-, inter-assay precision and accuracy, and LLoQ

Intra- and inter-assay precision and accuracy were assessed by replicate analysis (N = 5) of IQC solutions on the same day and in duplicate on different days (N = 5), respectively. Lower limits of quantification (LLoQ) were assessed by dilution of calibration solution 3 with analyte-free stripped human serum, and analysed in triplicate. The LLoQ was defined as the lowest concentration where inaccuracy and imprecision were < 20 %.

3.2.4.3 Extraction recovery, matrix effects, and process efficiency

To ascertain extraction recovery, matrix effect, and process efficiency ('overall recovery') the procedure suggested by Matuszewski *et al* (2003) was followed. All hepcidins (including internal standard) were added to analyte-free stripped human serum at the same concentration as IQCs A, B and C either before (A), or to analyte-free stripped human serum post extraction (B). All

hepcidins were also added to 35 % (*v/v*) aqueous acetonitrile containing 1 % (*v/v*) TFA (C). The peak area ratios (expressed as a percentage) of A to C, B to C, and B to A were calculated to ascertain process efficiency, matrix effects, and extraction recovery, retrospectively. Process efficiency was further ascertained by addition of all analytes (100 µg/L) to serum from 5 different sources (all analytes were below the LLoQ before fortification).

As hepcidin-25 has been suggested to be largely protein bound to α-2-macroglobulin, changes in the concentration of this protein - especially in those individuals with reduced kidney function, may affect the extraction of hepcidin-25,. Therefore, to ascertain whether there is an effect of increasing α-2-macroglobulin concentrations on process efficiency, α-2-macroglobulin was added to phosphate buffered saline containing all hepcidins (100 µg/L) at 1, 2, 3, and 4 g/L, and were analysed (N = 3) according to the protocol described herein. These concentrations of α-2-macrogobulin were chosen as they cover the range than can be expected in both healthy individuals and those with reduced kidney function (de Sain-van der Velden *et al.,* 1998).

3.2.4.4 Carry-over

Carry-over was assessed through consecutive analysis of prepared samples containing all analytes at low and high (L and H; 5 and 500 µg/L, respectively) concentrations in the order H, H, H, L, L, L.

3.2.4.5 Stability

3.2.4.5.1 Autosampler stability

To investigate analyte stability in the autosampler tray (10 ºC), separate solutions of hepcidin-20, -22, -24, and -25 were prepared in 20 % (*v/v*) aqueous acetonitrile containing 0.4 % TFA (*v/v*) at low (5 µg/L all analytes, except hepcidin-25: 10 µg/L), and high (50 µg/L all analytes, except hepcidin-25: 100 µg/L) concentrations. A portion of each sample was injected onto the LC-HR-MS every hour for up to 24 hours.

3.2.4.5.2 Freeze-thaw

Freeze–thaw stability was assessed by freezing (-20 ºC for 23 hours) and thawing (ambient room temperature, 1 hour) analyte-free stripped human serum containing individual hepcidins (50 µg/L). After thorough mixing, the solutions were analysed using freshly prepared calibration

solutions that had not undergone freeze-thawing. Portions of solutions that remained were returned to the freezer until the following day (3 freeze–thaw cycles in total).

3.2.4.5.3 Fortified and Clinical samples

To investigate analyte stability, all hepcidins were added to separate portions of analyte-free stripped human serum (final concentration 50 µg/L all analytes, except hepcidin-25 at 100 µg/L), and stored in the dark as approximately 500 µL portions in LoBind tubes at (i) -18 to -20 ºC, (ii) 2–8 ºC, and (iii) at ambient room temperature (approximately 20 °C). Each solution was sampled at 0, 1, 2, 4, 8 hours, 1 to 5, 7, 14, 21, and 28 days. Serum from 5 separate patients were also stored at (i) 2–8 ºC, and (ii) at ambient room temperature for 28 days. Each solution was sampled at time 0, after 1 to 3, 5, 7, 14, 21, and 28 days.

To ascertain whether there was degradation of any hepcidin isoform during the extraction process, each individual hepcidin was added to separate portions of analyte-free charcoal-stripped human serum (50 µg/L all analytes), and analysed in duplicate.

3.2.4.6 LC-HR-MS vs ELISA/LC-MS/MS method comparison

For method comparison purposes, 67, and 51 serum samples that had been previously analysed for hepcidin-25 using an ELISA, (Ganz *et al*., 2008), and a published LC-MS/MS method (Bansal *et al.,* 2010), respectively were used for method comparison purposes. All samples were stored at -20° for up to 12 months prior to analysis by LC-HR-MS.

3.2.4.7 Comparison of plasma and serum

For the comparison of all analytes in human plasma and serum, excess serum [Beckton Dickson (BD) Vacutainer SST II Advance] and plasma (BD Vacutainer dipotassium EDTA) from the same patient (N = 47) that had been submitted for routine biochemical and haematological analyses were collected from the Blood Sciences Laboratory, KCH. Samples were stored at 2–8 ºC for up to 4 hours before centrifugation (12,000 x *g*), and aliquots of the serum or plasma were stored in 0.5 mL LoBind tubes at -20 ºC until analysis. All aliquots were fully anonymised and all linkages with the sample to the patient removed.

3.2.5 Investigation of other hepcidin isoforms and oxidation products

Using Pinpoint software (ThermoFisher Scientific), theoretical *m/z* ratios and isotope patterns were generated for hepcidin-19, -21, and -23, and these were used to investigate whether other hepcidin isoforms maybe present in calibration/IQC solutions, or stability samples.

Hepcidin-24 and -25 both contain the amino acid methionine which is prone to oxidation to either a sulfone or sulfoxide. Theoretical *m/z* were generated using Pinpoint software and calibration solutions were retrospectively reviewed.

3.2.6 Charge state distribution

To investigate whether the distribution of charge state differs from sample-to-sample, data from serum samples used for comparison of plasma and serum were re-processed to provide peak areas of each individual charge state ($[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$), for each individual hepcidin.

3.2.7 Establishment of reference range

To establish a reference range for all hepcidin isoforms in healthy individuals using the current method, volunteers from the Pathology Laboratory, King's College Hospital, Denmark Hill, London, were requested to donate whole blood. All volunteers were given an information sheet and a consent form outlining the details of the request (Appendix A). After providing informed consent, sample was collected into BD Vacutainer SST II Advance blood collection tubes, and was fully anonymised by use of a participant identification number. Once collected, samples were immediately centrifuged (12,000 x *g*) and the serum separated. Serum ferritin, iron, c-reactive protein (CRP), and total iron binding capacity were immediately measured. Serum ferritin (LLoQ: 1 µg/L) was measured using a direct chemiluminometric two-site immunoassay (Siemens Healthcare Diagnostics Ltd, UK). C-reactive protein (LLoQ: 2.0 mg/L) was measured with an ELISA (Wako Chemicals, Germany). Total iron binding capacity (TIBC, LLoQ: 1 µmol/L) was calculated indirectly by the unsaturated iron binding capacity method. Serum iron was measured using a colourimetric assay (LLoQ: 1 µmol/L). Normal reference ranges for these analytes used within KCH were as follows; ferritin 20–300 (males), 20–200 (females) µg/L, CRP < 5 mg/L, TIBC (males and females) 50–72 µmol/L, and TSAT (males and females) 20–50 %. Following analysis, serum was stored in separate 0.5 mL LoBind tubes at -20 ºC within 2 hours

of collection until analysis for hepcidin isoforms. Samples were analysed for all hepcidin isoforms within 1 week of storage.

3.2.8 Statistical analysis

Statistical analysis was performed using Analyse-it (Analyse-it Software Ltd, Leeds, UK) for Microsoft excel, and SPSS for Windows, version 23.0 (SPSS Incorporated, Chicago, USA). Normality of data distribution was assessed using the Shapiro–Wilk test. Relationships were explored using Spearman's rank correlation (r), the Mann–Whitney U test or Kruskal-Wallis test. A p value < 0.05 was considered statistically significant. To determine predictors of serum hepcidin isoform concentrations, univariate regression analysis was first undertaken, and variables where p = > 0.1 were excluded from further multivariate regression analysis. To correct for skewness, hepcidin isoform concentrations were log transformed prior to univariate and multivariate regression analysis.

3.3 Results

3.3.1 Method validation

3.3.1.1 Calibration and mass accuracy

Calibration was linear (R^2 > 0.94) for all analytes over the range studied (Figure 3-1). Extracted ion chromatograms together with analyte retention times, and mass spectrums are shown in Figure 3-2, Figure 3-3, and Figure 3-4, respectively. The LC peak width at half height for all analytes was approximately 3 seconds; with approximately 12 points under each peak (unsmoothed data, calibration solution 4). Mean (SD) intra- (N = 5), and inter-assay (N = 5) mass error for all analytes was + 0.13 (1.32), and -0.68 (1.64) ppm, respectively.

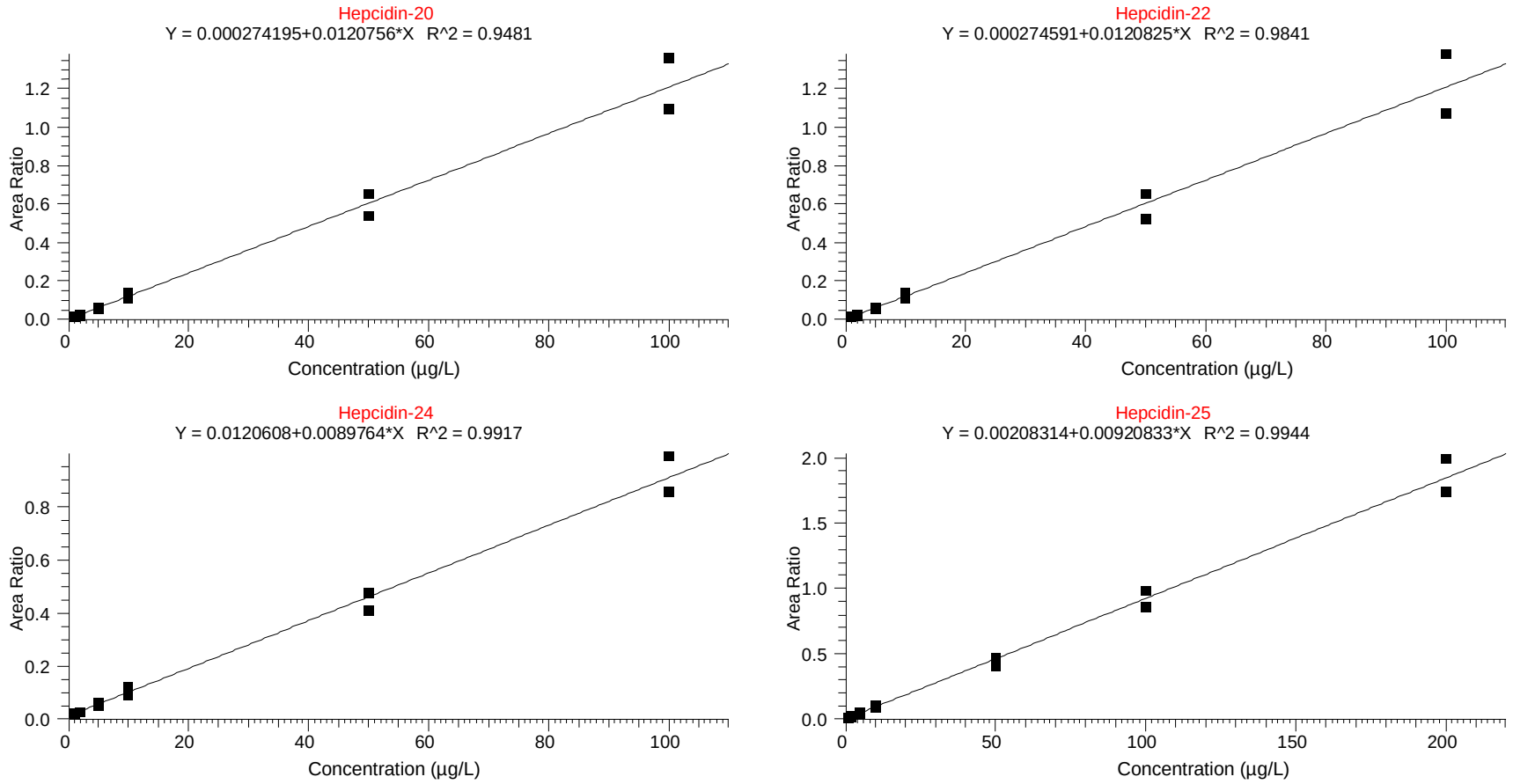


Figure 3-1 Calibration curves for hepcidin-20, hepcidin-22, hepcidin-24, and hepcidin-25. Samples extracted in duplicate from stripped human serum and analysed using developed LC-HR-MS method.

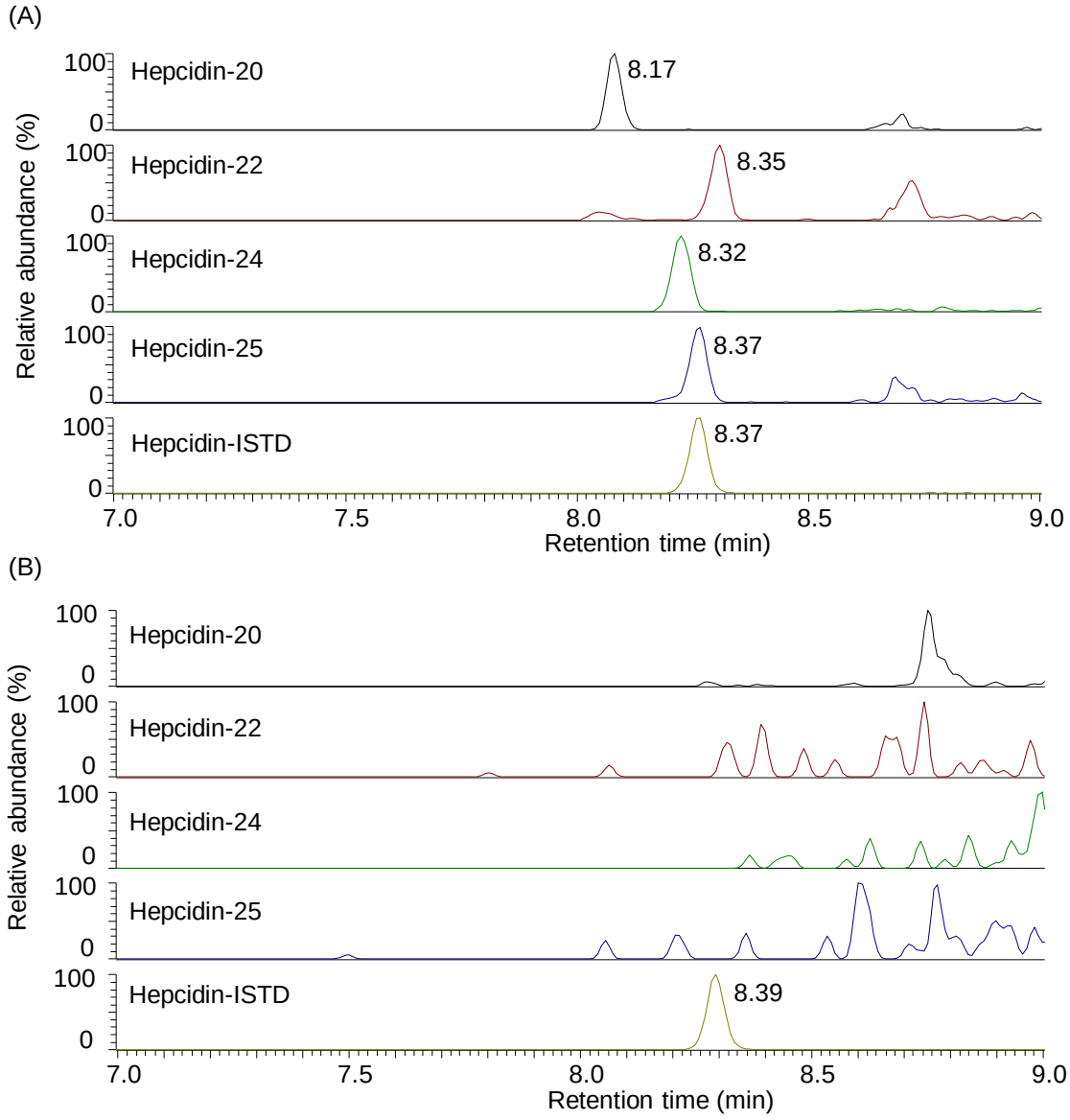


Figure 3-2 Typical extracted ion chromatogram showing analyte retention time. Extraction window ± 10 ppm based on theoretical *m/z*. Chromatograms: (A) calibration standard 4 (10 µg/L, all analytes), and (B) analyte-free stripped human serum.

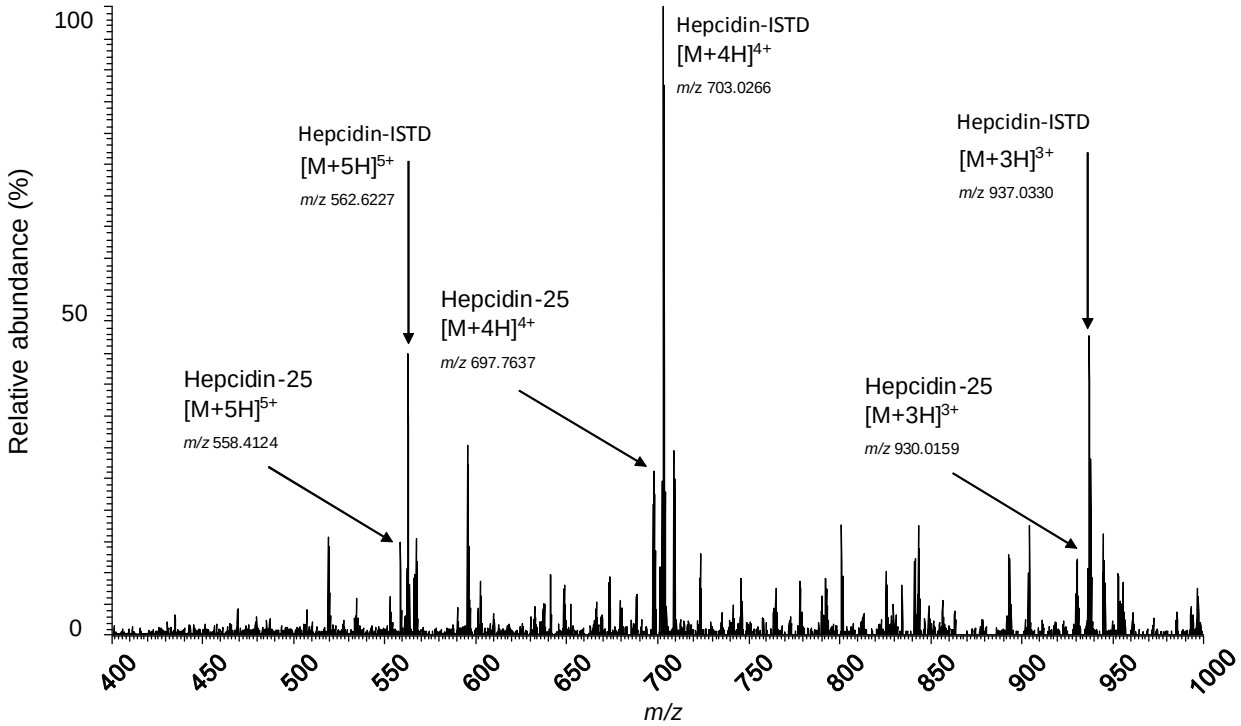


Figure 3-3. Mass spectrum of a calibration standard 4 (10 µg/L, all analytes), indicating charge state and theoretical monoisotopic masses for hepcidin-25 and internal standard.

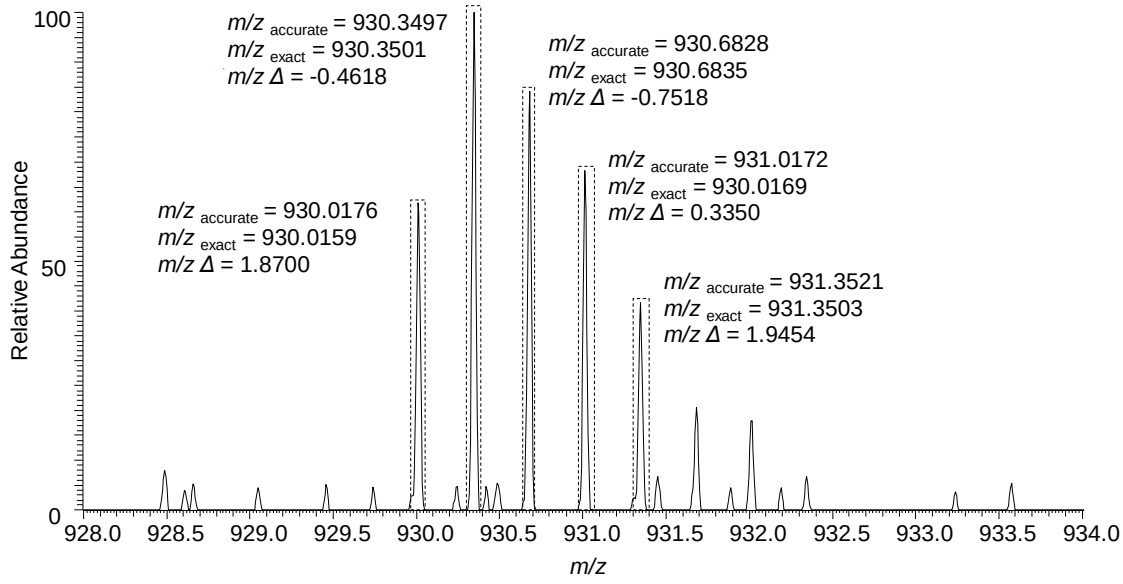


Figure 3-4. Mass spectrum of the $[M+3H]^{3+}$ of hepcidin-25, from a calibration solution 4 (10 µg/L). The top 5 isotopes monitored are indicated together with a mass extraction window of ± 10 ppm (dashed line). The accurate mass, exact mass and the mass error (*m/z Δ*, parts per million) are given for each isotope.

3.3.1.2 Intra-, inter-assay precision and accuracy, and LLoQ

Intra- and inter-assay precision and accuracy are summarised in Table 3.2. The LLoQ was 1 µg/L for all analytes.

Table 3.2. Summary accuracy and precision data.

	Analyte											
	Hepcidin-20			**Hepcidin-22**			**Hepcidin-24**			**Hepcidin-25**		
Intra-assay (N = 5)												
Nominal concentration (µg/L)	4	8	25	4	8	25	4	8	25	8	75	150
Mean measured concentration (µg/L)	4	9	26	4	8	24	5	9	30	9	80	164
RSD (%)	11	14	6	< 1	6	7	11	10	9	9	2	6
Accuracy (% nominal)	100	113	104	100	100	96	125	113	120	113	107	109
Inter-assay (N = 5)												
Nominal concentration (µg/L)	4	8	25	4	8	25	4	8	25	8	75	150
Mean measured concentration (µg/L)	4	9	26	4	8	25	5	9	29	8	78	154
RSD (%)	21	6	14	4	2	6	22	13	15	6	7	7
Accuracy (% nominal)	100	113	104	100	100	100	125	113	116	100	100	103

3.3.1.3 Extraction recovery, matrix effects, and process efficiency

Extraction recovery, matrix effects, and process efficiency for all analytes are summarised in Table 3.3. Median (range) process efficiency for all analytes added to analyte-free serum from 5 separate sources were; 36 (22–45), 25 (18–37), 23 (17–26), 24 (15–38), and 23 (15–32) % for hepcidin-20, -22, -24, -25, and -ISTD, respectively. Increasing the concentration of α-2-macroglobulin had no effect on process efficiency ($p = 0.23$ to 0.52 for all analytes, Figure 3-5).

Table 3.3. Extraction efficiency, matrix effects, and process efficiency for all analytes at low (hepcidin-20, -22, -24: 4 µg/L, hepcidin-25: 8 µg/L), medium (hepcidin-20, -22, -24: µg/L, hepcidin-25: 75 µg/L), and high (hepcidin-20, -22, -24: 25 µg/L, hepcidin-25: 150 µg/L) concentrations when added to stripped human serum. Mean of triplicates, SD in parentheses.

	Low IQC				
	Hepcidin-20	**Hepcidin-22**	**Hepcidin-24**	**Hepcidin-25**	**Hepcidin-ISTD**
Extraction efficiency	24 (11)	20 (7)	18 (7)	17 (6)	16 (6)
Matrix effects	109 (22)	130 (25)	132 (27)	133 (26)	132 (25)
Process efficiency	25 (7)	25 (5)	22 (4)	22 (4)	20 (4)
	Medium IQC				
	Hepcidin-20	**Hepcidin-22**	**Hepcidin-24**	**Hepcidin-25**	**Hepcidin-ISTD**
Extraction efficiency	49 (19)	53 (22)	40 (15)	50 (21)	49 (20)
Matrix effects	69 (10)	83 (14)	104 (17)	95 (16)	96 (16)
Process efficiency	35 (16)	44 (7)	41 (6)	47 (7)	47 (7)
	High IQC				
	Hepcidin-20	**Hepcidin-22**	**Hepcidin-24**	**Hepcidin-25**	**Hepcidin-ISTD**
Extraction efficiency	35 (13)	47 (12)	42 (8)	43 (10)	42 (10)
Matrix effects	68 (6)	78 (8)	90 (8)	84 (7)	84 (8)
Process efficiency	23 (6)	36 (7)	37 (6)	36 (7)	35 (7)

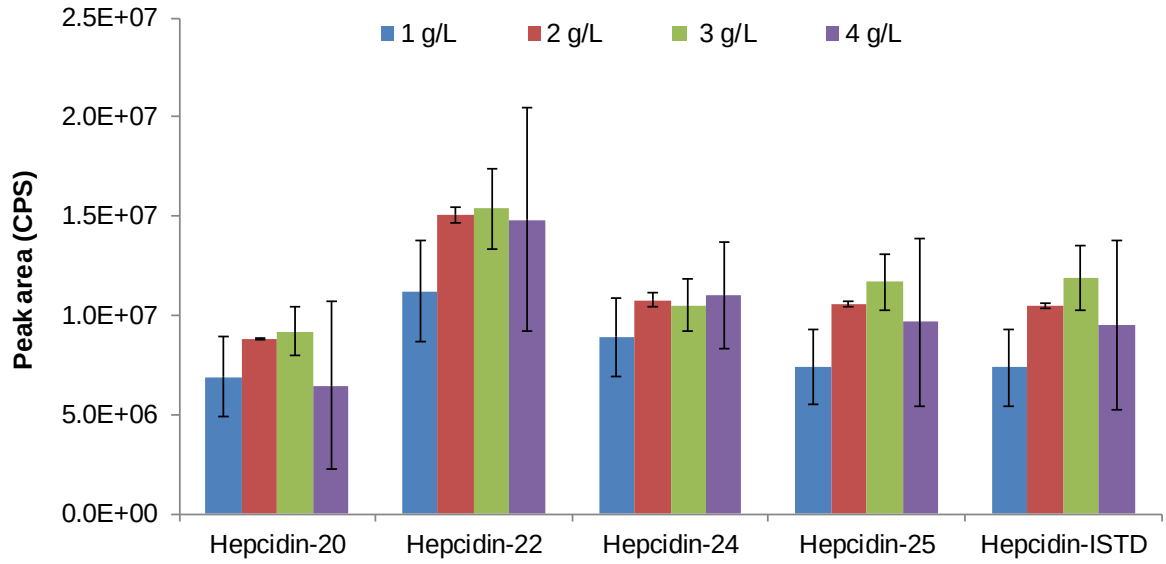


Figure 3-5. Effect of increasing α-2-macroglobulin concentrations on peak areas of all hepcidins. Mean of triplicates, error bars represent ± standard deviation (Kruskal-Wallis; hepcidin-20, p = 0.46; hepcidin-22, p = 0.42; hepcidin-24, p = 0.52; hepcidin-25, p = 0.25; hepcidin-ISTD, p = 0.23).

3.3.1.4 Carry-over

No significant carry-over was observed for any analyte

3.3.1.5 Stability

3.3.1.5.1 Autosampler stability

All analytes were stable in extracts on the autosampler at low and high concentrations respectively (Figure 3-6 and Figure 3-7).

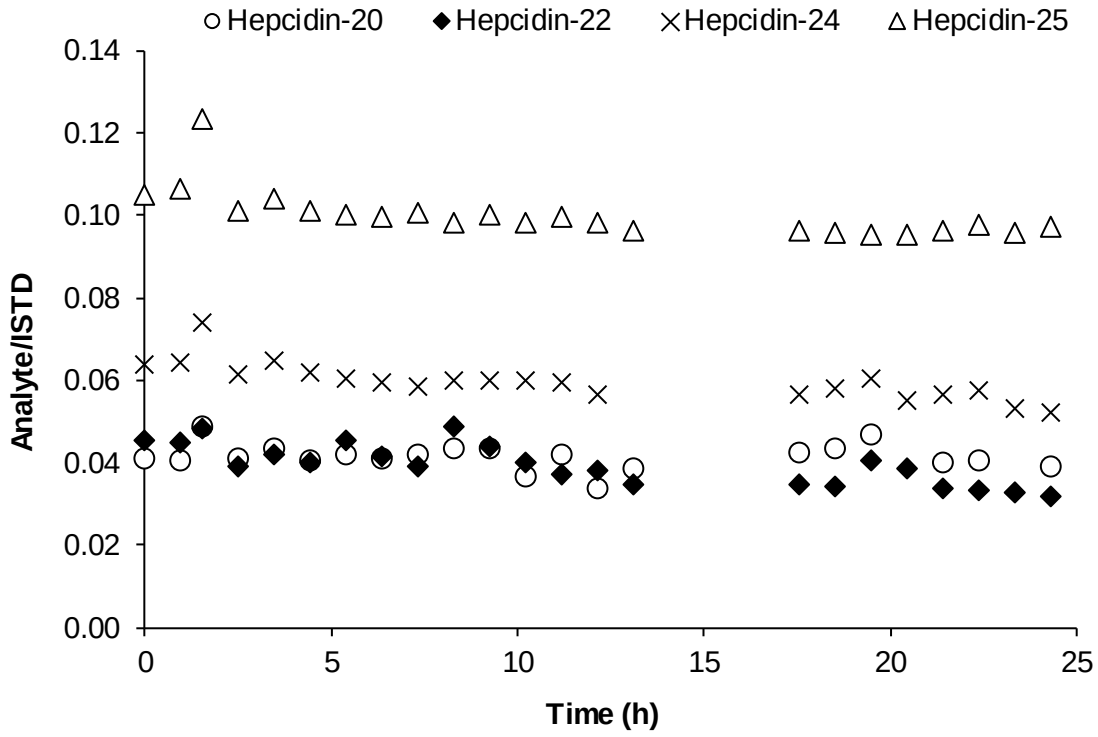
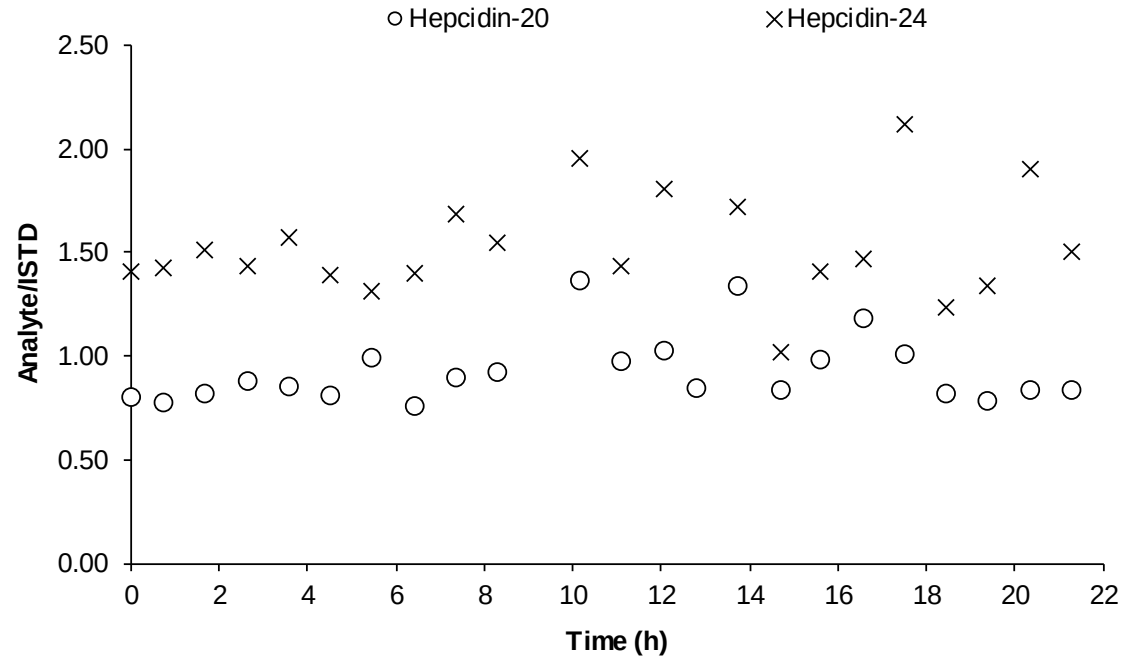


Figure 3-6 Stability of hepcidin-20, -22, -24 and, -25 in extracts on the autosampler at 10 ºC over 25 hours when corrected for the internal standard. (Gap in analysis due to autosampler error)

(A)



(B)

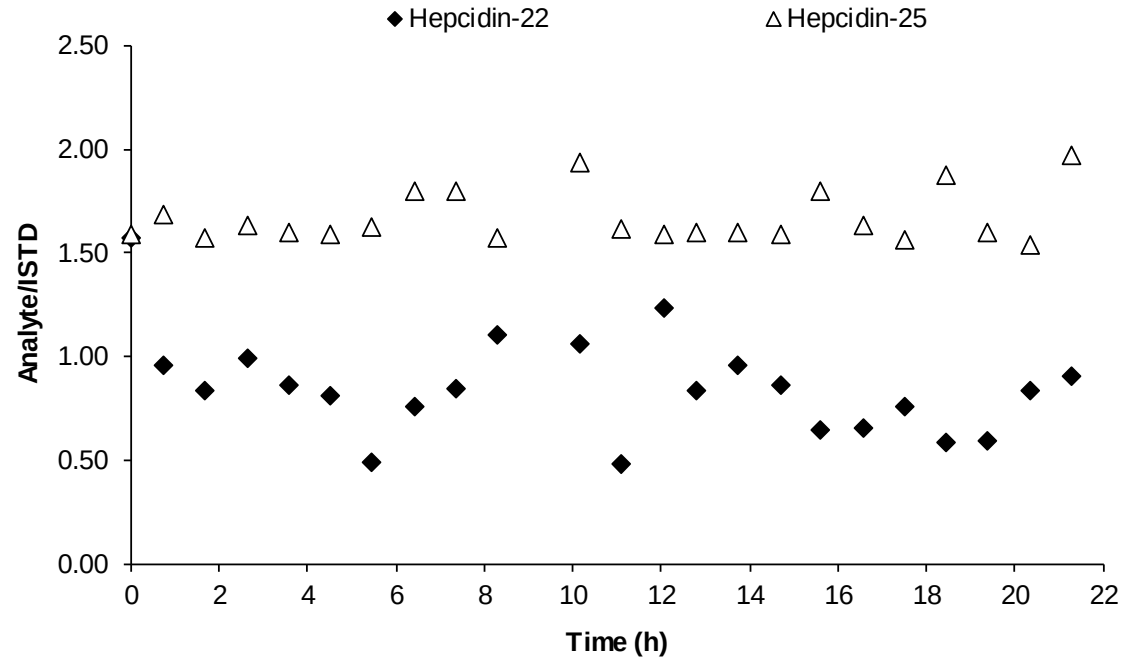


Figure 3-7 Stability of (A) hepcidin-20, and -24, and (B) hepecidin-22, and -25 in extracts on the autosampler at 10 ºC over 22 hours when corrected for the internal standard.

3.3.1.5.2 Freeze-thaw

All analytes were stable following 3-freeze thaw cycles

3.3.1.5.3 Fortified samples

When stored at ambient room temperature all analytes were stable (± 20 % of initial concentration) for 1 week, after which there was a steady decline until week 4 where the concentration of hepcidin-20 was only 15 % of that measured initially, and for hepcidin-22, 24, and -25; 40 % of that initially. All analytes were stable for up to 3 weeks at 2–8 ºC, after which concentrations began to decline (Table 3.4). Under all storage conditions the decline in hepcidin-20, -24, and -25 concentrations was not paralleled by an increase in the concentrations of other known hepcidin isoforms. However, the decline in hepcidin-22 when stored at room temperature was paralleled by the presence of increasing concentrations of hepcidin-20 (Figure 3-8). Hepcidin-20 was also detected after day 2 when hepcidin-22 was stored at 2–8 ºC, although measured concentrations were low (median 3, range 2–6 µg/L). There was no identifiable degradation or formation of hepcidin-20, 22, 24, or 25 during the extraction process.

Table 3.4. Analyte stability when added to separate portions of stripped human serum, and stored at; (A) ambient room temperature in the dark, and; (B) 2–8 ºC. * Due to sampling error data not available.

(A)

Time			Analyte concentration (%[a]) after storage at ambient room temperature			
Hours	**Days**	**Weeks**	**Hepcidin-20**	**Hepcidin-22**	**Hepcidin-24**	**Hepcidin-25**
1	-	-	84	95	96	91
2	-	-	80	101	102	98
3	-	-	93	96	100	106
4	-	-	90	*	84	*
8	-	-	86	*	91	94
24	1	-	95	109	86	89
48	2	-	126	98	69	90
72	3	-	83	89	80	90
96	4	-	76	93	91	86
120	5	-	85	90	85	93
168	7	1	77	81	82	89
336	14	2	42	62	65	95
504	21	3	32	51	49	*
672	28	4	15	42	38	44

(B)

Time			Analyte concentration (%[a]) after storage at 2–8 ºC			
Hours	**Days**	**Weeks**	**Hepcidin-20**	**Hepcidin-22**	**Hepcidin-24**	**Hepcidin-25**
1	-	-	74	100	96	93
2	-	-	98	101	95	97
3	-	-	80	101	71	94
4	-	-	*	103	*	105
8	-	-	61	111	103	101
24	1	-	91	97	95	97
48	2	-	60	109	96	98
72	3	-	59	99	86	99
96	4	-	103	105	76	91
120	5	-	60	93	84	92
168	7	1	85	98	90	100
336	14	2	81	99	82	93
504	21	3	77	99	86	100
672	28	4	69	*	70	74

a Results percentage of analyte concentration at time '0' hours

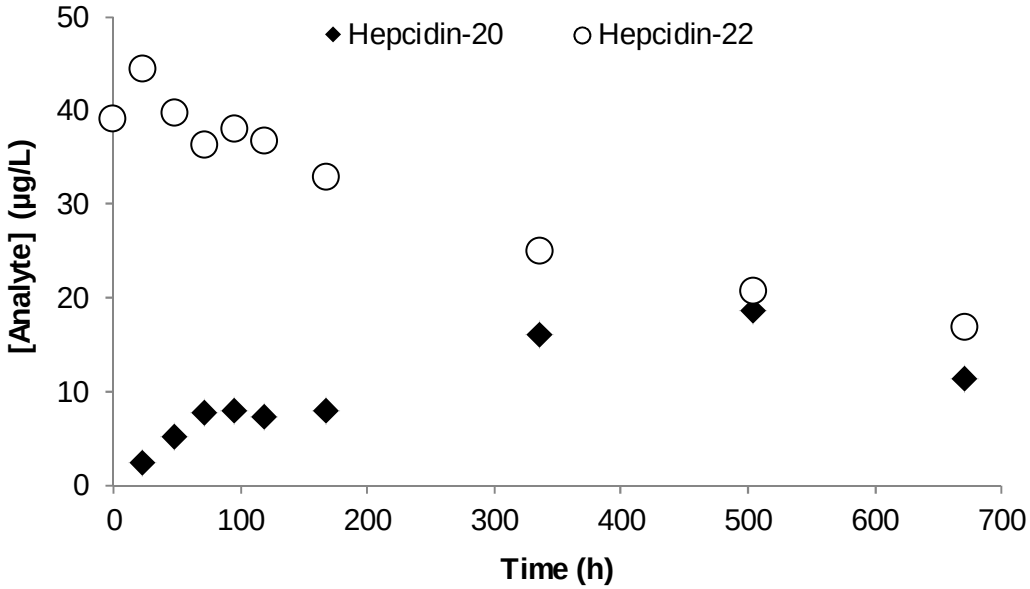


Figure 3-8. Stability of hepcidin-22 when added to stripped human serum, and stored at 2–8 ºC, showing an increase in the presence of hepcidin-20.

3.3.1.5.4 Clinical samples

When stored at ambient temperature the concentration of all analytes steadily declined day-to-day, by broadly the same amount (Figure 3-9). Although hepcidin-25 was stable (± 20 % of initial concentration) in samples 2 and 3 at the third day, in the remaining samples concentrations had declined to 70–80 % of those at day 1.

When stored at 2–8 ºC all analytes were considerably more stable than at room temperature; hepcidin-25 was stable (± 20 % of initial concentration) for 5–7 days, however there was again much variability between samples (Figure 3-10).

In all samples stored either at room temperature, or at 2–8 °C there was no evident increase in hepcidin-20 -22, or -24 that was associated with a decline in hepcidin-25 concentrations.

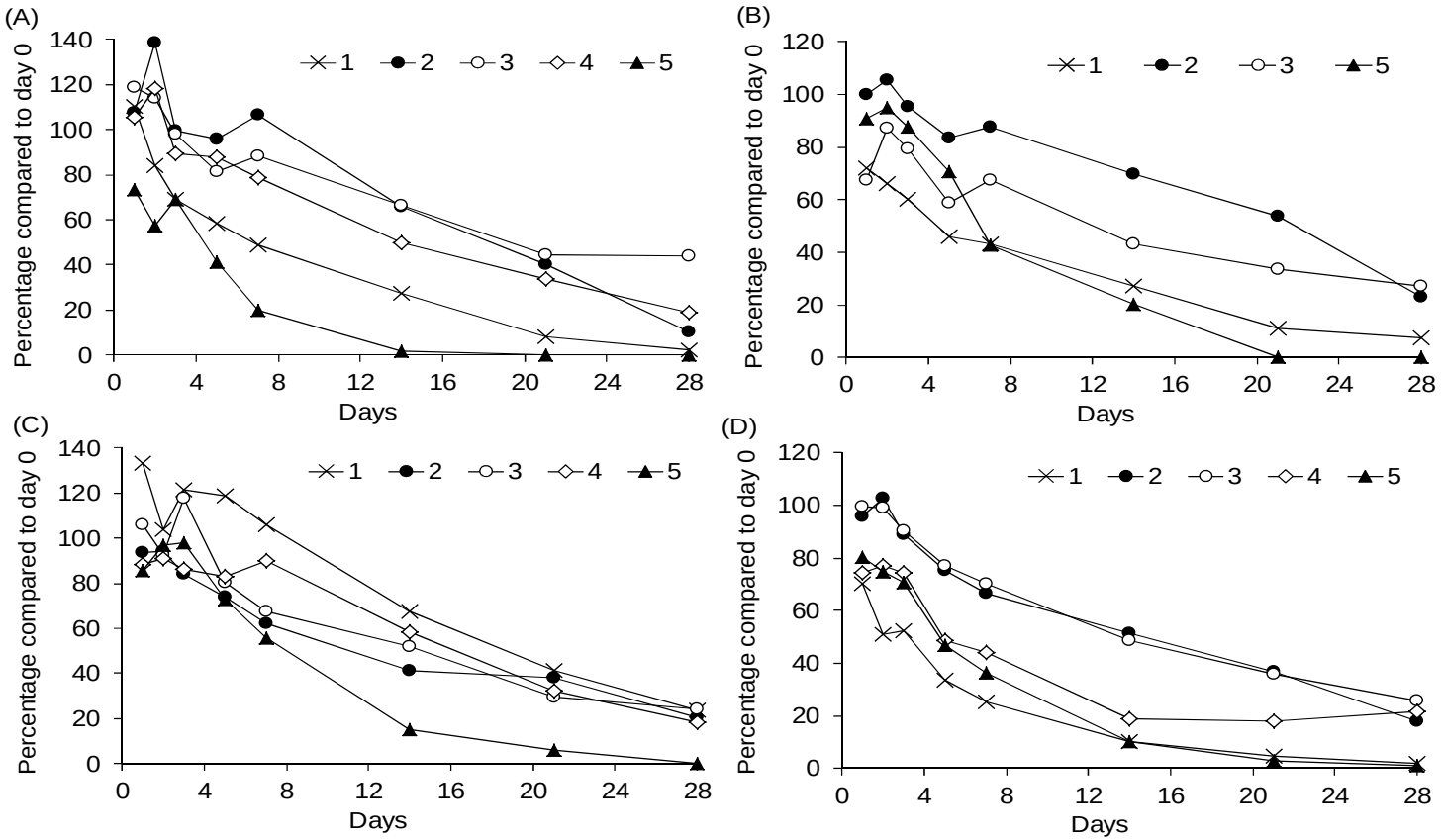


Figure 3-9 Stability of hepcidin isoforms in five serum patient samples when stored in the dark at ambient room temperature for up to 28 days. (A) Hepcidin-20, (B) hepcdidin-22, (C) hepcidin-24, and (D) hepcidin-25. Data expressed as a percentage of the concentration measured initially. Initial concentrations in samples 1–5 for hepcidin-20; 3, 9, 6, 1, 6 µg/L, hepcidin-22; 4, 16, 7, < 1, 4 µg/L, hepcidin-24; 17, 19, 12, 6, 19 µg/L, and hepcidin-25; 57, 72, 79, 15, and 112 µg/L, respectively.

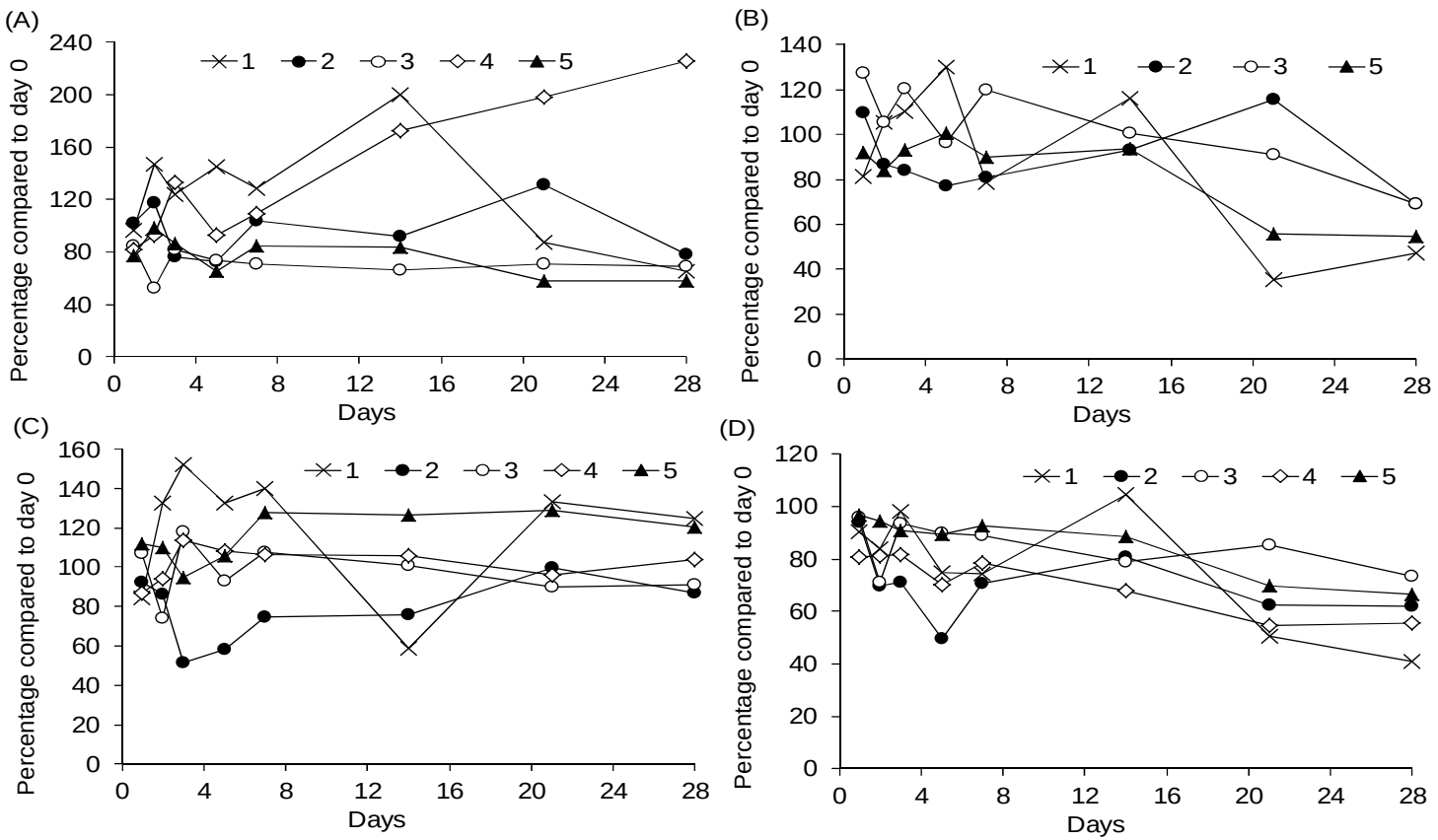


Figure 3-10. Stability of hepcidin isoforms in five patient samples when stored at 2–8 °C for up to 28 days. (A) Hepcidin-20, (B) hepcdidin-22, (C) hepcidin-24, and (D) hepcidin-25. Data expressed as a percentage of the concentration measured at time '0'. Concentrations at time 0 in samples 1–5 for hepcidin-20; 2, 7, 5, 1, 3 µg/L, hepcidin-22; 3, 16, 4, < 1, 4 µg/L, hepcidin-24; 17, 17, 9, 4, 15 µg/L, and hepcidin-25; 63, 71, 92, 14, and 125 µg/L, respectively.

3.3.1.6 Method comparisons

3.3.1.6.1 LC-HR-MS vs ELISA

A scatterplot, and Bland-Altman plot of the sample comparison is shown in Figure 3-11. There was a positive and significant correlation ($r = 0.79$, $p < 0.0001$) for hepcidin-25 between the two methods used. However, the concentrations of hepcidin-25 measured by the developed LC-HR-MS method were approximately 13 % of those measured using the ELISA method, and with proportional ($p < 0.0001$) bias. In no sample was either hepcidin-20, or hepcidin-22 measured. In 10 samples hepcidin-24 was detected, but the concentration never exceeded 2 µg/L.

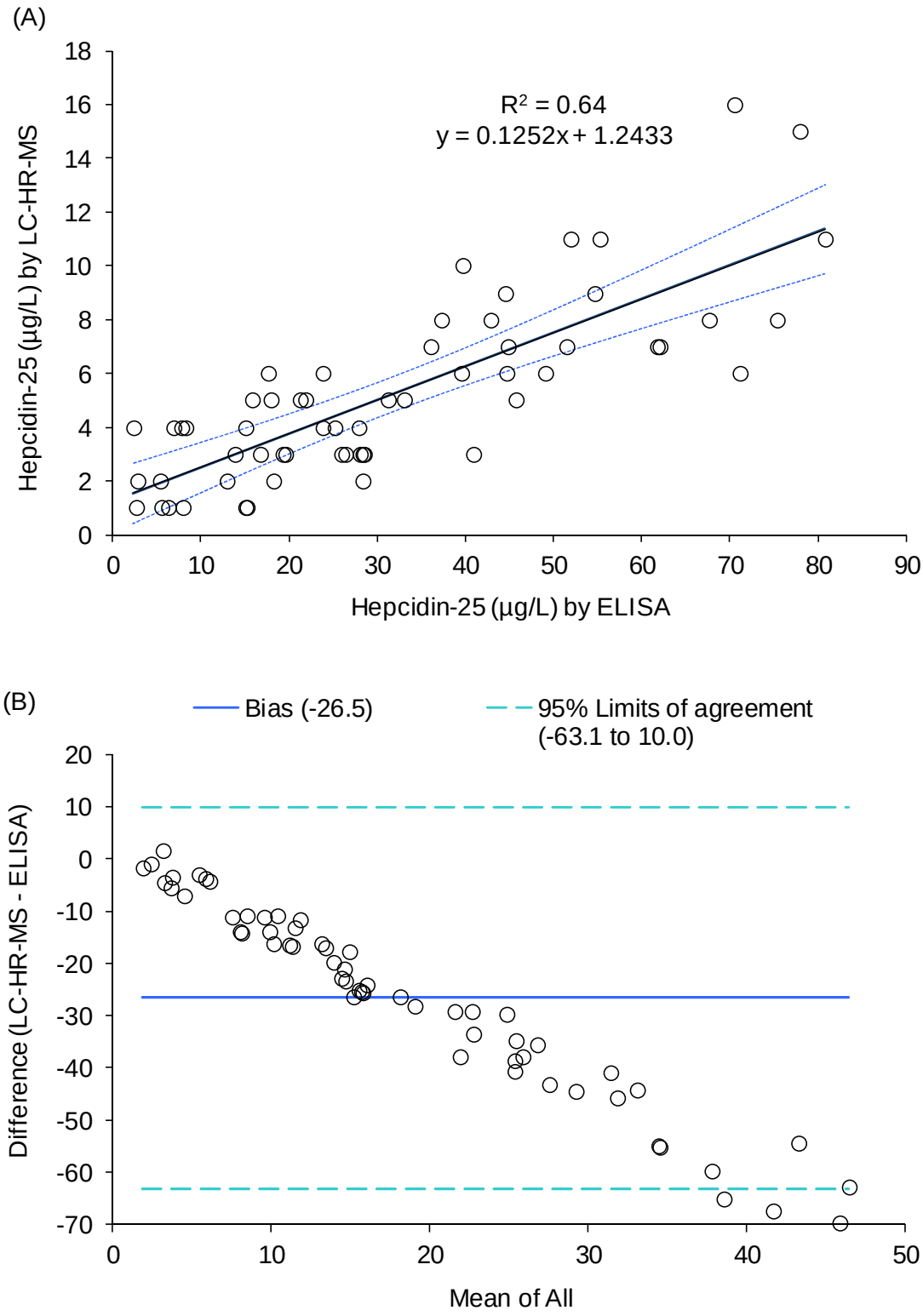


Figure 3-11. Scatterplot (A), and Bland-Altman plot (B) showing the correlation between serum hepcidin-25, in samples when analysed using an ELISA and the developed LC-HR-MS method. Solid line = simple linear regression, dashed lines = 95 % limits of agreement.

3.3.1.6.2 LC-HR-MS vs LC-MS/MS

A scatterplot, and Bland-Altman plot of the sample comparison is shown in Figure 3-12. There was a very good correlation between the two methods for hepcidin-25, although there was a bias that was proportional ($p < 0.05$).

Hepcidin-20, -22, and -24 were detected in 86, 69, and 69 % of samples, respectively, and the median (range) hepcidin-20, -22, and -24 concentrations measured were 11 (1–91), 2 (1–23), and 3 (1–62) µg/L, respectively. The published LC-MS/MS method used for comparison did quantify hepcidin-20, -22, or -24.

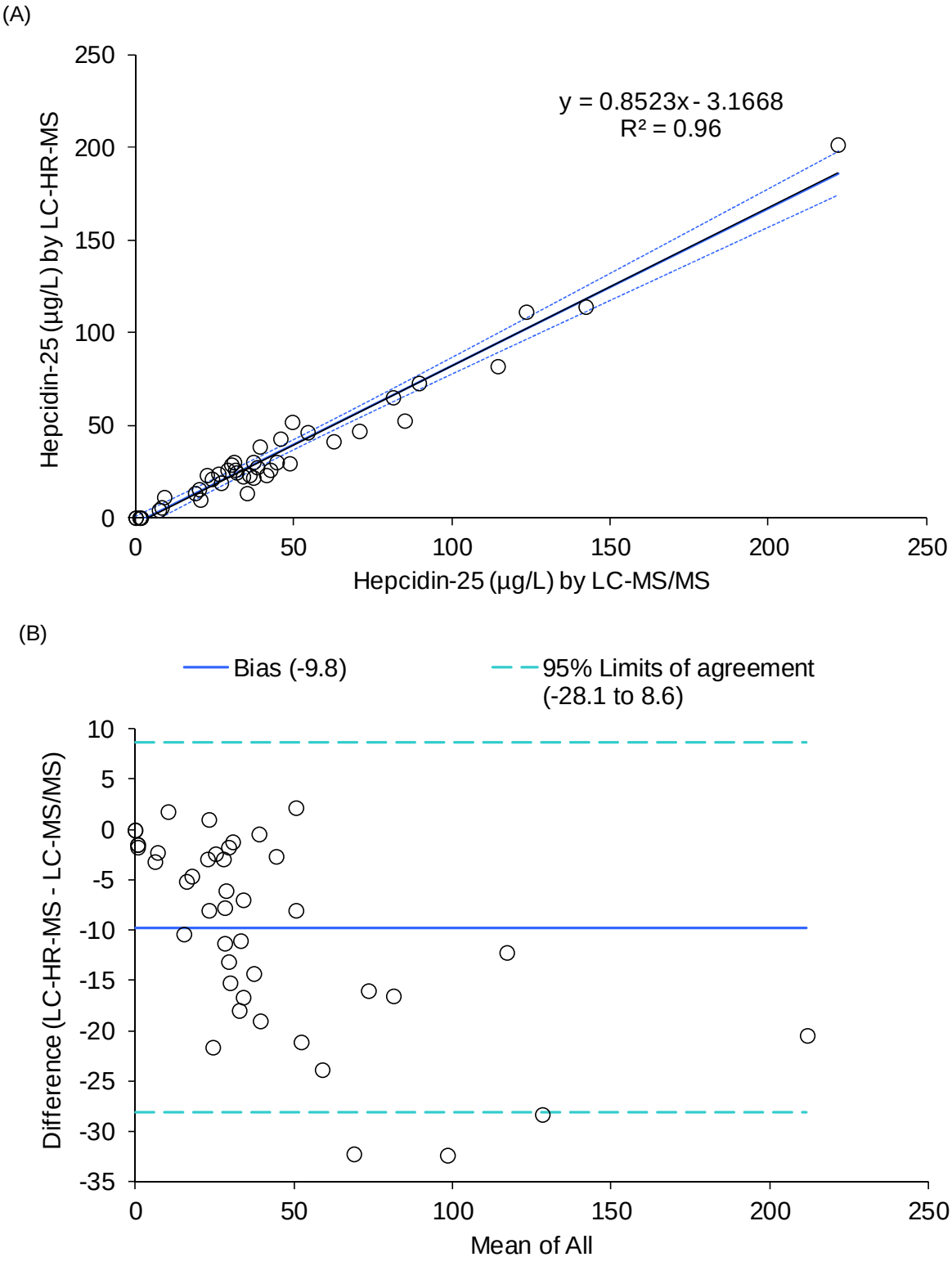


Figure 3-12. Scatterplot (A), and Bland-Altman plot (B) showing the correlation between serum hepcidin-25 in samples (N = 45) when analysed using a published LC-MS/MS method, and the developed LC-HR-MS method. Solid line = simple linear regression, dashed lines = 95 % limits of agreement.

3.3.1.7 Comparison between plasma and serum

There was a strong and significant correlation ($r = \geq 0.95$, $p = < 0.05$) between all analytes in paired plasma and serum (Figure 3-13). Hepcidin-20, -22, 24, and -25 were detected in 74 (N = 35), 23 (N = 11), 53 (N = 25), and 89 (N = 42) % of serum samples. Median (range) serum hepcidin-20, -22, -24, and -25 concentrations measured were 4 (1—34), 6 (2—21), 8 (1—51), 46 (1—353), respectively.

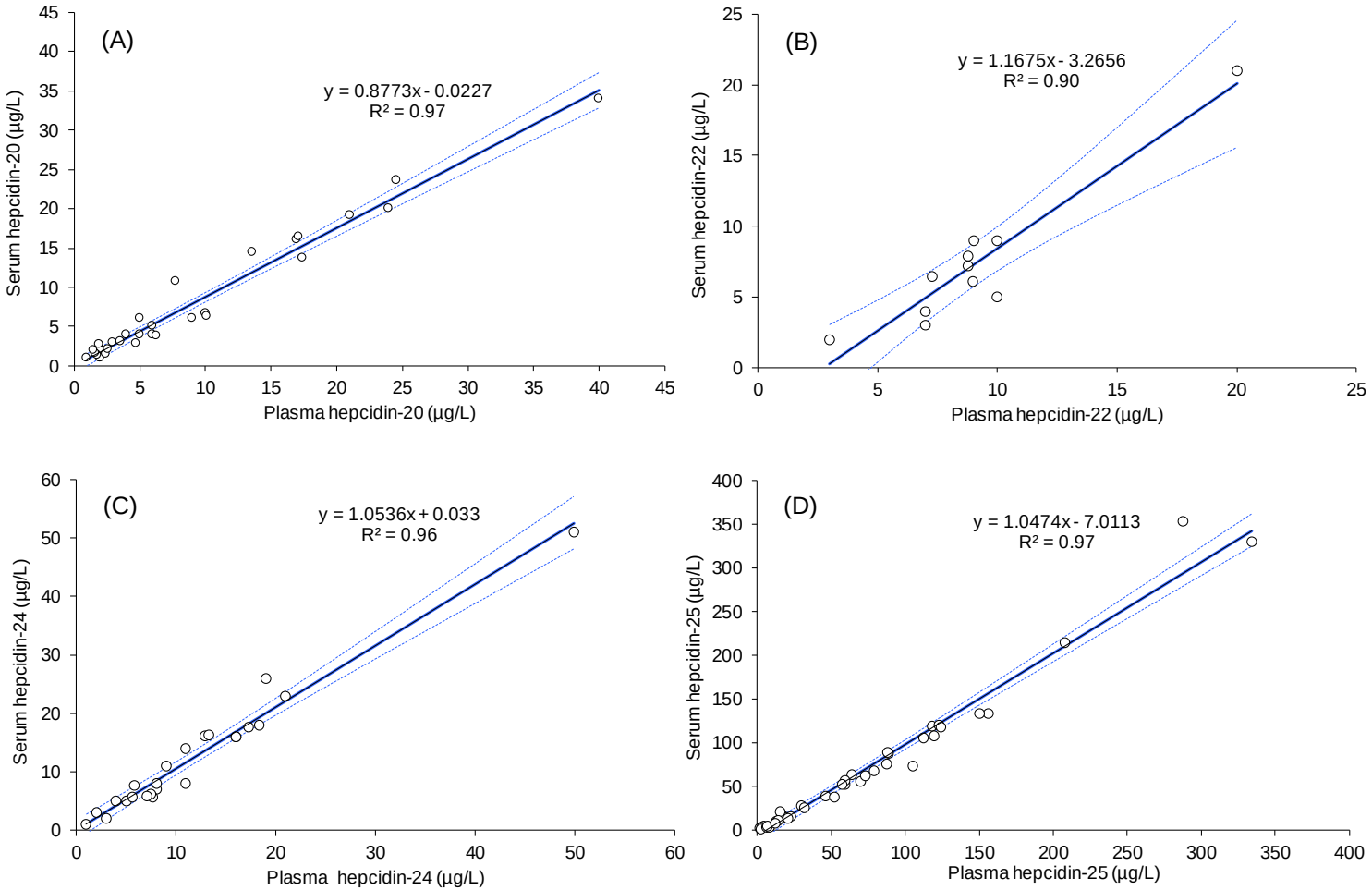


Figure 3-13. Scatterplots of (A) hepcidin-20 (N = 35); (B) hepcidin-22 (N = 11); (C) hepcidin-24 (N = 25); and (D) hepcidin-25 (N = 42) in human plasma and serum. Dashed line = 95 % confidence intervals, Solid line = simple linear regression.

3.3.2 Investigation of other hepcidin isoforms and oxidation products

Neither hepcidin -21, or -23 were identified in any calibration or IQC solution, however, there was a small peak present at the same retention time as hepcidin-20 (8.18 min, area 0.4 % of the hepcidin-20 peak), in those samples fortified with hepcidin-20. Reviewing the mass spectrum of this peak identified ions corresponding to the theoretical masses of the $[M+4H]^{4+}$ charge state of hepcidin-19 (*m/z* 520.1790, 520.4300, and 520.6790). This was also consistent with the tentative identification of hepcidin-19 in the hepcidin-20 tuning solution. Sulfone/sulfoxide products of hepcidin-24 and hepcidin-25 were not identified.

3.3.3 Charge state distribution

The contribution of each charge state to the sum of the $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$ charge states are shown in Table 3.5.

Table 3.5. Percentage contribution of each individual charge state to the sum of $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$. Data shown as mean (SD).

	Charge state		
	$[M+3]^{3+}$	**$[M+4]^{4+}$**	**$[M+5]^{5+}$**
Hepcidin-20	65.4 (4.6)	31.2 (4.6)	3.2 (4.6)
Hepcidin-22	44.7 (5.2)	47.8 (5.2)	6.5 (5.2)
Hepcidin-24	44.9 (9.2)	42.5 (9.2)	9.8 (9.2)
Hepcidin-25	33.4 (10.5)	52.7 (10.5)	13.0 (10.5)
Hepcidin-ISTD	37.2 (12.3)	48.7 (12.3)	13.8 (12.3)

For hepcidin-20, the $[M+3H]^{3+}$ ion is most abundant, whereas for hepcidin-22, -24, -25 and the internal standard the charge is broadly shared between the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ charge states. This distribution of charge state is very different to that obtained when each analyte was individually infused into the mass spectrometer (Figure 2-1, and Figure 2-2).

3.3.4 Establishment of reference range

Serum was collected from 59 volunteers, 21 were male [median (range) age; 39 (24–68) yr], and 38 female [median (range) age: 36 (21–61) yr]. Ethnicity was recorded as white 3(6 individuals), black (11), mixed race (2), Asian (7), and 3 did not provide this information. Forty-

six recorded no medication, 5 reported as being on the oral contraceptive pill, and the remainder were on miscellaneous drugs (e.g. omeprazole, insulin, tetracyclizine). No medical conditions were mentioned of any note, except for 2 females that had β-thalassemia. For comparison of data, and for assessment of reference ranges, individuals with β-thalassemia were excluded (N = 2), as were those with a ferritin concentration < 20 µg/L (N = 11), and those where hepcidin-25 was below the LLoQ (N = 5).

Summary biochemical data and serum hepcidin-25 concentrations measured are given in Table 3.6. Ferritin, and transferrin saturation were significantly lower in females as compared to males (p = < 0.01, and < 0.05, respectively). The median (range) hepcidin-25 concentration measured was 8 (1–31 µg/L), and despite median hepcidin-25 concentrations being lower in females (6 µg/L) as opposed males (11 µg/L), this was not statistically significant (p = 0.22). There was no significant difference between males and female for CRP (p = 0.56), iron (p = 0.12), TIBC (p = 0.08), ferritin:hepcidin-25 (p = 0.31), or TSAT:hepcidin-25 (p = 0.47). The median (range) hepcidin-25: ferritin ratio was 14.8 (3.9–75.7), and the TSAT:hepcidin-25 ratio was 3.0 (5.3–21.0). Of those samples where hepcidin-25 was not detected, ferritin, iron, TIBC, and TSAT were all within their reference ranges, and were not indicative of iron deficiency. Hepcidin-20, -22, and -24 were below the LLoQ (1 µg/L) in all patient samples.

Table 3.6. Summary biochemical data from (A) male, and (B) female serum volunteer samples (N.B. hepcidin-20, -22, and -24 were < 1 µg/L in all samples analysed).

(A) Males (N = 17)

	Mean	Minimum	10th percentile	Median	90th percentile	Maximum
Ferritin (µg/L)	133	51	55	101	222	348
C-reactive protein (mg/L)	2.2	< 2.0	3.2	3.0	6.0	9.7
Iron (µmol/L)	17.9	8.8	13.8	16.3	24.2	31.7
Total iron binding capacity (µmol/L)	57	49	51	56	64	67
Transferrin saturation (%)	32	16	22	31	43	63
Hepcidin-25 (µg/L)[1]	10	2	2	11	17	20
Ferritin:Hepcidin-25	18.7	3.8	5.9	14.3	29.6	82.9
TSAT:Hepcidin-25	5.4	1.4	1.7	2.9	13.9	21.9

(B) Females (N =24)

	Mean	Minimum	10th percentile	Median	90th percentile	Maximum
Ferritin (µg/L)	83	20	24	61	193	311
C-reactive protein (mg/L)	2.1	< 2.0	2.3	2.9	3.1	3.2
Iron (µmol/L)	15.9	7.3	10.1	15.4	24.1	29.0
Total iron binding capacity (µmol/L)	59	46	54	58	66	70
Transferrin saturation (%)	27	12	17	27	41	44
Hepcidin-25 (µg/L)[2]	8	1	1	6	16	31
Ferritin:Hepcidin-25	14.9	3.1	4.6	9.7	29.4	69.3
TSAT:Hepcidin-25	7.3	0.9	1.4	4.0	17.1	39.3

[1] Excludes 3 samples where hepcidin-25 < 1 µg/L

[2] Excludes 2 samples where hepcidin-25 < 1 µg/L

Hepcidin-25 was positively correlated with ferritin (r = 0.48, p = < 0.05, Figure 3-14), and negatively correlated with TIBC (r = -0.40, p = < 0.05, Figure 3-15). Hepcidin-25 was not correlated with serum iron (r = 0.05, p = 0.76, Figure 3-14) or transferrin saturation (r = 0.16, p = 0.32, Figure 3-15). Multivariate regression analysis, including the predictors ferritin and TIBC, only explained 26 % of the variation in serum hepcidin-25 concentrations, and showed ferritin (p = < 0.05) to be the only significant, predictor of hepcidin-25 concentrations.

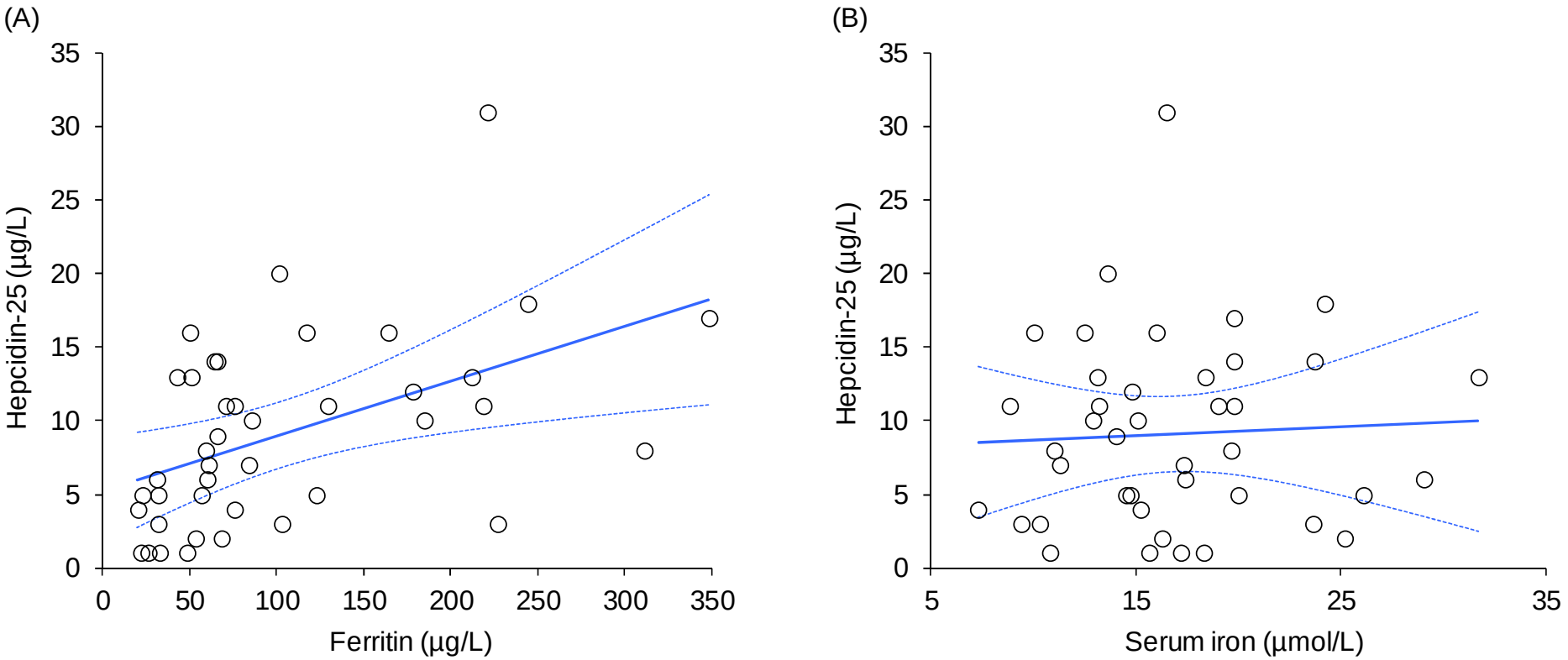


Figure 3-14. Scatterplots showing the correlation between serum hepcidin-25, and (A) ferritin, and (B) iron in healthy volunteers. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

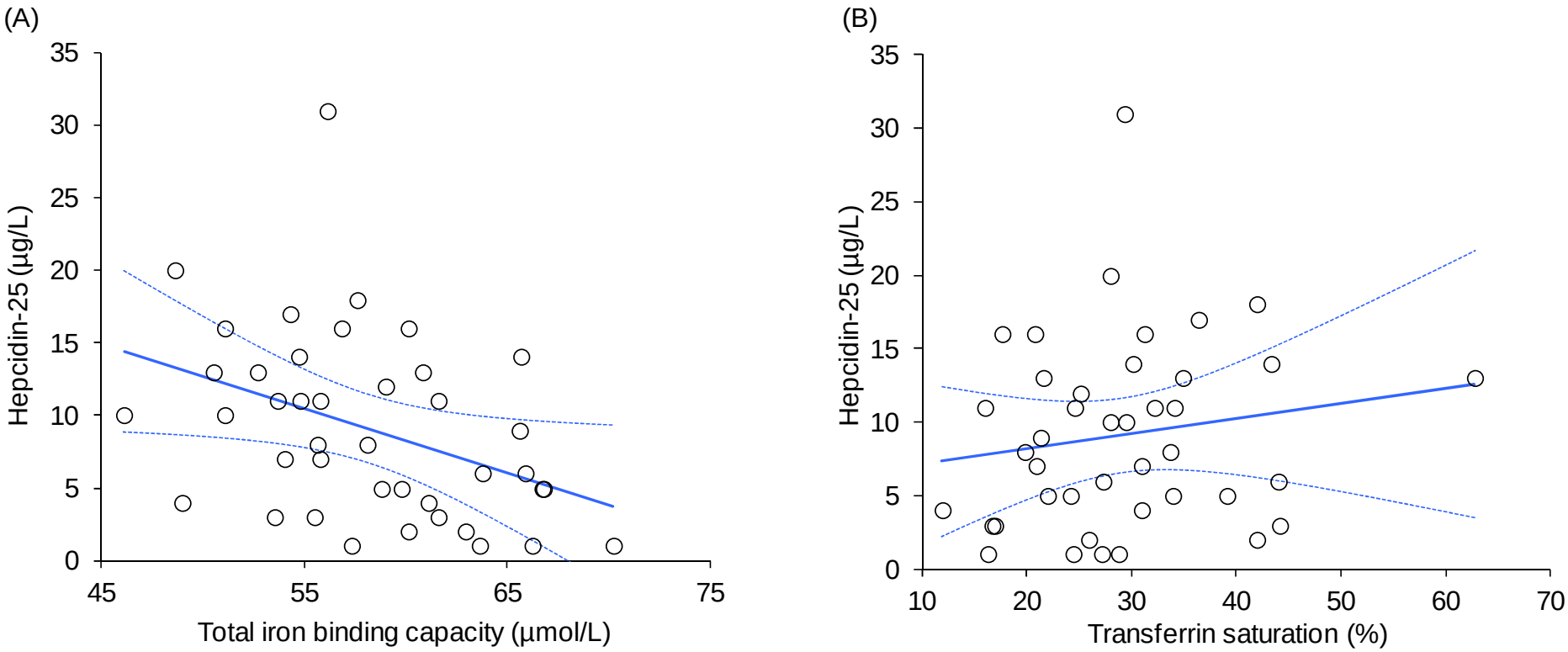


Figure 3-15. Scatterplots showing the correlation between serum hepcidin-25, and (A) TIBC ferritin, and (B) iron, (C) TIBC, and (D) transferrin saturation, in healthy volunteers. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

3.4 Discussion

3.4.1 Method validation

The method developed here has good sensitivity, and is selective, with acceptable accuracy and precision for all hepcidin isoforms. Although, for hepcidin-20, and -24 precision at the lowest IQC (4 µg/L) was marginally outside the acceptance criteria of 20 %, It was still acceptable. There were minimal matrix effects for all analytes, and process efficiency, whilst low (< 50 %, all analytes), was compensated for by the internal standard, and an LLoQ of 1.0 µg/L was still achieved for all analytes.

3.4.1.1 Stability and oxidation products

All hepcidins (except hepcidin-25) were stable at ambient room temperature for 5–7 days (hepcidin-25; 3 weeks) when added to stripped human serum. However, in patient samples stored at ambient room temperature, concentrations of all hepcidin isoforms had begun to decline by day 1. These latter findings are broadly in-keeping with published reports that have found hepcidin-25 to be stable for 16 h in patient samples, when stored at room temperature (Li *et al.,* 2009). All hepcidin isoforms in both stripped human serum, and patient samples were more stable when stored at 2–8 °C.

It is not clear why hepcidin isoforms appeared to be more stable at ambient room temperature when added to stripped human serum, as compared to patient samples. However, the process of charcoal stripping, that removes some hormones and steroids, may remove enzymes involved in the degradation of hepcidin isoforms. Certainly, the treatment of foetal bovine serum with charcoal has been shown to reduce enzyme activity, electrolytes, and certain enzymes (Cao *et al.,* 2009).

It is interesting to note that when added to stripped human serum, the decline in hepcidin-24, and -25 concentrations was not paralleled by measurable concentrations of the smaller *N*-truncated isoforms of these analytes. Whereas, the decline in hepcidin-22 was paralleled by an increase in hepcidin-20 when stored at ambient room temperature. In patient samples, there was also no evident increase in any hepcidin isoform concentration that coincided with the decline in hepcidin-25.

Hepcidin-19, -21, or -23 were also not identified as being formed during storage, although this can only be tentatively concluded as without a reference compound, the chromatographic retention time, nor whether these compounds are extracted under the current methodology cannot be elucidated. But as hepcidin-19, -21, and -23 have very similar physiochemical properties (Table 1.1) to those hepcidins investigated here, it would be feasible to assume that they would extract, and ionise in a similar way.

Since there was no evident degradation of each hepcidin isoform to a smaller form, this would suggest that the decline during storage in hepcidin isoforms is likely attributable to adsorption onto the container wall or endogenous compounds, or due to peptide aggregation, which has been reported for hepcidin-25 (Malyszko, 2009).

Hepcidin-24 and -25 both contain the amino acid methionine which is prone to oxidation to either a sulfone or sulfoxide, and the oxidation of both hepcidin-24 and -25 have been identified in urine samples (Swinkels *et al*., 2008). Oxidation of hepcidin-24 and -25 in serum has been reported to be minimal (Ward *et al*., 2008; Swinkels *et al*., 2008), and is supported by the findings here.

3.4.1.2 Method comparison

3.4.1.2.1 LC-HR-MS vs ELISA

There was a positive and significant correlation for hepcidin-25 between the LC-HR-MS method developed here and a commercially available ELISA. However, concentrations of hepcidin-25 measured using LC-HR-MS were some 10 fold lower than those obtained when using the ELISA. Variability between immunochemical and chromatographic techniques have been reported in a number of 'round robins' (Kroot *et al.,* 2009; Kroot *et al.,* 2012; van der Vorm *et al.,* 2016), and it has been suggested that part of this variability could be due to the presence of hepcidin isoforms other than hepcidin-25 present in samples, causing interference in immunochemical assays. However, this is certainly not the cause of the 10 fold difference in hepcidin-25 concentrations measured here, as all samples used in the comparison had non-detectable or very low concentrations of hepcidin-20, -22, or -24. It is likely that the cause of this disparity is in the preparation of calibration solutions used in the ELISA assay, or that the antibody used in the ELISA is cross-reacting with an unknown endogenous compound. Another investigator (Itkonen *et al*., 2012) reported no correlation at all with the DRG ELISA used here

when comparing it to an LC-MS/MS based assay for hepcidin-25. Furthermore, a review by Kroot *et al*., (2011) states that *"the bioactive hepcidin kit of DRG Instruments, (purchased October 2009) gave similar concentrations for all samples and could not discriminate between iron metabolism disorders (unpublished results)"*. It should be remembered though, that the samples used here for comparison purposes were stored for up to 12 months at -20 °C following initial analysis by ELISA and stability data covering this period is not available. Therefore, lack of analyte stability may compromise the data here. However, hepcidin-25 has been shown to be stable for up to 2 years following storage at -80 °C (Laarakkers *et al*., 2013).

3.4.1.2.2 LC-HR-MS vs LC-MS/MS

The excellent correlation between the method developed here and a previously published LC-MS/MS assay helps support the assumption that the assay developed is fit for purpose. Furthermore, the LC-MS/MS assay used for comparison purposes has reported to perform well in previous round robins (Sukhi Bansal, personal communication).

3.4.1.3 Comparison of plasma and serum

There was an excellent correlation between EDTA plasma and serum for all analytes, which is in keeping with other reports for hepcidin-25 (Kobold *et al.,* 2008; Butterfield *et al.,* 2010). Other investigators have reported a bias of some 24 % for hepcidin-25 between human EDTA plasma and human serum (Lefebvre *et al.,* 2015). Lithium-heparin plasma and serum are reported to be comparable (Busbridge *et al.,* 2009; Itkonen *et al.,* 2012), although other investigators have reported marginally higher concentrations (8 %) of hepcidin-25 in EDTA plasma compared to heparinised plasma (Laarakers *et al.,* 2013). There have been no reports as to the correlations of hepcidin-20, -22, or -24 between human plasma and serum, other than that described here, where there was a good correlation between these two matrices. This would indicate, certainly for the method developed here, that should serum not be available for hepcidin isoform quantitation, then EDTA plasma is a suitable alternative. However, since routine biochemical tests for assessing iron status (i.e. ferritin, TSAT) are undertaken on serum this maybe the most suitable sample type for hepcidin measurement, as it would allow all markers of iron status to be measured on a single sample.

3.4.2 Charge state distribution

Distribution of the charge state for all hepcidins was significantly different in patient samples as compared to that observed when each analyte was separately infused into the mass spectrometer. This may not be that surprising as unknown compounds (e.g. proteins, phospholipids, and drugs) present in human serum, which are not present in the infusion solution, are likely to affect ionisation of the analyte within the MS source. As the method developed here acquires data in full-scan mode, and the area of the three charge states are summed, any variability in isotope and/or charge state distribution is unlikely to affect accurate quantitation. A disadvantage to this approach is that monitoring a number of isotopes and charge states can reduce the signal to noise ratio, and therefore reduce sensitivity. However, a LLoQ of 1.0 µg/L was still achieved for all analytes, which is comparable to reported LC-MS/MS methods for hepcidin-25 (Murphy *et al.,* 2007; Kobold *et al.,* 2008; Wolff *et al.,* 2013). Published LC-MS/MS methods for hepcidin-25 acquire data in SRM mode using triple quad mass-spectrometers, monitoring a single charge state and there is no uniformity in the charge state being monitored, with some methods monitoring the $[M+3H]^{3+}$ (Wolff *et al.,* 2013; Kobbold *et al.,* 2008), $[M+4H]^{4+}$ (Li *et al.,* 2009; Hwang *et al.,* 2011), or the $[M+5H]^{5+}$ ion (Delaby *et al.,* 2014). It is possible that the most appropriate pre-cursor and product ions of a given charge state are not being monitored in these methods, since the distribution of charge state may differ between that seen when infusing the solution into the MS and when analysing samples; this could affect accurate quantitation. It is advisable that if hepcidin isoforms are to be quantified by LC-MS/MS, at least two charge states, and ideally 3 should be monitored to account for variability between samples.

3.4.3 Establishment of reference range

The concentrations of hepcidin-25 measured here are broadly comparable with those reported by other investigators (Addo *et al.,* 2016; Anderson *et al.,* 2011; Bansal *et al.,* 2010; Campostrini *et al.,* 2012; Galesloot *et al.,* 2011; Hwang *et al.,* 2011; Kemna *et al.,* 2007; Kroot *et al.,* 2010). Although, significantly higher hepcidin-25 concentrations have been reported by some investigators (Ganz *et al.,* 2008; Koliaraki *et al.,* 2009). In these reports though, immunochemical assays have been used, and it is likely that there is some cross-reactivity of

hepcidin-20, -22, and -24 with the hepcidin-25 antibody used, thereby falsely elevating the reported hepcidin-25 concentration.

Mean (95 % CI) serum hepcidin-20 concentrations of 7.24 (6.94–7.58) µg/L have been reported in one large study (N = 1,577) of the general population (Campostrini *et al.,* 2012). However, in only half (54.2 %) of all samples was hepcidin-20 detected. In another study, mean serum hepcidin-20, and -22 concentrations of 2.6 and 0.97 µg/L were measured in 40 healthy volunteers (Addo *et al.,* 2015). Hepcidin-24 concentrations in healthy controls have not been reported to date. Neither hepcidin-20, -22, or -24 were at detectable concentrations in the volunteer samples measured here, this may simply be because they are not present in the small number of samples analysed, or they may be present but below the detection limits of the assay. Certainly, the concentrations of hepcidin-22 reported by Addo *et al.,* 2015, were below the LLoQ of the method developed here. It should also be noted in the report by Addo *et al.,* 2015, no details were given as to assay validation or the source of the reference compounds used, thereby raising concerns over the validity of the concentrations reported.

For hepcidin-25, age and sex specific methodological reference ranges should always be established, as well as the ratio of transferrin saturation to hepcidin-25, and ferritin to hepcidin-25. A limitation of the work undertaken here is the relatively small number of samples obtained for ascertaining method dependent reference ranges, and a lack of standardisation with regards to when the samples were taken. Be this as it may, a method based reference range for hepcidin-25 has been established.

3.5 Conclusion

Presented here is the appropriately validated LC-HR-MS method for the measurement of all commercially available hepcidin isoforms in human serum. There was a good correlation ($R^2 \geq 0.90$) between dipotassium EDTA plasma and serum for all analytes, and a good correlation with a published LC-MS/MS method. In samples from healthy volunteers (N = 46), hepcidin-20, -22, and -24 were below the LLoQ (1 µg/L) in all samples, and a reference range for hepcidin-25 of 1–31 µg/L has been ascertained (excludes 5 samples < 1 µg/L).

Chapter 4 Hepcidin in Chronic Kidney Disease

4.1 Introduction

Chronic kidney disease (CKD) is a term that refers to the progressive loss (over months or years) of renal function. CKD is contrary to acute kidney injury, which is sudden damage to the kidney, usually as a consequence of another serious illness (e.g. heart failure). The severity of CKD is defined by the calculated estimated glomerular filtration rate (eGFR) (Table 4.1), with a normal eGFR being above 90 mL/min/1.73 m^2. Using data from the combined 2009 and 2010 Health Survey for England, it has been estimated that 6 % of men and 7 % of women have an eGFR below 60 mL/min/1.73 m^2, and therefore reduced kidney function (Aitken *et al.,* 2014).

Table 4.1. Stages of CKD based on estimated glomerular filtration rate.

Stage	eGFR (mL/min/1.73 m^2)	Description
1	> 90	Normal kidney function but urine findings or structural abnormalities or genetic trait point to kidney disease
2	60–89	Mildly reduced kidney function, and Other findings (as for stage 1) point to kidney disease
3a	45–59	Moderately reduced kidney function
3b	30–44	Moderately reduced kidney function
4	15–29	Severely reduced kidney function
5	< 15 or on dialysis	Very severe, or end stage kidney failure

Causes of CKD include the various types of glomerulonephritis, chronic tubulo-interstial disease such as chronic pyelonephritis, genetic disease such as polycystic kidney disease, immune-mediated disease such as systemic lupus erythematosus or vasculitis, and long-term use of some medications (e.g. non-steroidal anti-inflammatory drugs, proton pump inhibitors, lithium, etc), however, high blood pressure or diabetes are the most common causes. Anaemia is a well-known complication of CKD, and estimates have suggested that some 12 % of patients with CKD in the UK have anaemia [defined as a haemoglobin concentrations ≤ 12 g/dL in women and ≤ 13 g/dL in men (Mikhail *et al.,* 2012; WHO, 1968)]. The major cause of anaemia in these patients is reduced erythropoietin (EPO) production, which decreases with increasing severity of kidney damage. Therefore, treatment with erythropoiesis-stimulating agents (ESAs) is a common treatment strategy (NICE, 2015). However, in some 10–20 % of patients with anaemia, treatment with ESAs is ineffective, with one of the most common causes of ESA hypo-responsiveness being iron deficiency (Babitt & Lin, 2012). Iron-deficiency may be ‘absolute’ (i.e.

when the total body iron stores are exhausted), or 'functional' (where there are ample iron stores but a failure to release iron rapidly enough to satisfy the demands of the bone marrow (Macdougall *et al.,* 1989). Haemodialysis patients are particularly prone to absolute iron deficiency from chronic bleeding, frequent phlebotomy, or from blood trapping in the dialysis apparatus, and it has been estimated that patients can lose some 1–3 g of iron per year (Babitt & Lin, 2012).

Treatment with iron supplementation is a recommended treatment in non-dialysing CKD patients. With the choice of either oral or intra-venous (IV) iron depending on the severity of iron deficiency, availability of preparations, and previous response to oral iron therapy (KDIGO, 2012). Management of iron deficiency in patients with CKD is complicated by the poor response in some patients to oral iron, particularly those on haemodialysis with functional iron deficiency. Therefore, it is recommended that IV iron should be used in all patients with CKD undergoing dialysis (KDIGO, 2012). However, the use of IV iron is more costly than oral iron when accounting for drug, administration, and indirect costs to the patient for time and travel, and it can also cause rare but serious short-term effects including anaphylactic-type reactions, hypotension, and arthralgia (Liles, 2012).

Hepcidin-25 has been recognised as having a key role in functional iron deficiency and anaemia in CKD (Macdougall *et al*., 2010). Poor gastro-intestinal absorption of dietary/supplementary oral iron is proposed to be due to raised plasma concentrations of hepcidin-25 as a result of inflammatory cytokines (e.g. IL-6, known inducers of hepcidin-25 transcription), although reduced eGFR, and therefore reduced excretion may be a contributing factor. Inappropriately raised plasma hepcidin-25 concentrations are also believed to be a contributing factor to the persistent anaemia present in those patients treated with ESAs, and as such, therapeutics targeting the hepcidin-25-ferroportin axis are currently being investigated. One such therapeutic, Lexaptepid pegol, a pegylated structured *L*-oligoribonucleotide that binds and inactivates hepcidin-25, has been shown to inhibit hepcidin-25, and raise serum iron concentrations and TSAT in patients with anaemia of chronic disease (Boyce *et al.,* 2016). It is feasible that this compound may work in patients with anaemia of CKD. It is also interesting to note that in cell-lines, vitamin D directly inhibits hepcidin-25 transcription, and a pilot study in healthy human volunteers showed a decrease in serum hepcidin-25 within 24 hours of a single dose of 25D-hydroxyvitamin D (Bacchetta *et al.,* 2014).

Measuring hepcidin-25 concentrations may be of use in the clinical assessment of anaemia in CKD patients, and in guiding the selection of which therapy is most likely to be successful in individual patients. Additionally, since patients with CKD have raised hepcidin-25 concentrations, it is likely that *N*-truncated isoforms of hepcidin-25 are also present, although few studies have measured hepcidin-20, and -22 in patients with CKD, and no studies to date have reported concentrations of hepcidin-24. This is of particular concern since hepcidin-25 'specific' immunoassays are still being widely used for hepcidin-25 measurement in these patients, and it is likely that there is cross-reactivity of hepcidin-20, -22, and -24 with the hepcidin-25 antibody used, hence reported hepcidin-25 concentrations may be misleading. Furthermore, even though *N*-truncated isoforms of hepcidin-25 have been shown to have little if any activity at the FP-1 receptor, they may have a role as a biomarker for various diseases, and they may have some antimicrobial activity (Ho *et al.,* 2013).

The aims of this chapter are as follows:

- Measure hepcidin isoforms in serum samples from patients with CKD not undergoing haemodialysis, as well as those individuals undergoing haemodialysis.
- Investigate the impact of haemodialysis on the concentrations of hepcidin isoforms.
- Investigate relationships of hepcidin isoform concentrations with demographic and clinical variables using univariate and multivariate regression analysis
- Identify potential use and discuss role of hepcidin isoform measurements in chronic kidney disease

4.2 Methods

4.2.1 Patient samples

Patients with varying stages of CKD (not requiring dialysis) attended a renal clinic at King's College Hospital, London for routine monitoring. A whole blood sample (BD Vacutainer dipotassium EDTA), and a serum (BD Vacutainer SST II Advance) sample was taken for routine biochemical and haematological tests (including ferritin, CRP, TIBC, iron, creatinine, and haemoglobin) as part of their on-going care. Patients with CKD undergoing haemodialysis had a serum sample taken immediately before the procedure, and again directly afterwards, for

routine biochemical tests as part of their on-going care. Full blood count and biochemical tests were undertaken within the Blood Sciences Laboratory of King's College Hospital, in accordance with local procedures (Chapter 3.2.7).

After completion of all biochemical tests, excess serum was stored into 1.5 mL protein LoBind tubes at -20 ºC for up to one month until analysis for hepcidin isoforms. All samples were taken as a part of routine care, excess sample was fully anonymised, and all linkages with the sample to the patient removed, therefore review by a research ethics committee was not required, in accordance with Royal College of Pathologists, and Human Tissue Act Guidelines (Marks, 2012, Human Tissue Authority, 2017). Time taken between collection of the sample and storage for hepcidin measurement was approximately 6 hours, during this time the sample was stored at 2–8 ºC.

Samples obtained from healthy volunteers that were used to establish a reference range for hepcidin isoforms (Chapter 3.3.4) were used as controls for comparison purposes.

4.2.2 Analytical methods

Hepcidin isoforms were measured in singlicate, with a set of calibration and IQC solutions at the beginning and end of each assay and an IQC every 10 samples using the LC-HR-MS method previously described.

4.2.3 Statistical analysis

Statistical analysis was performed using Analyse-it (Analyse-it Software Ltd, Leeds, UK) for Microsoft excel, and SPSS for Windows, version 23.0 (SPSS Incorporated, Chicago, USA). Normality of data distribution was assessed using the Shapiro–Wilk test. Relationships were explored using Spearman's rank correlation (r), the Mann–Whitney U (grouped data), or the Wilcoxon signed-rank (matched pairs) test. A p-value < 0.05 was considered statistically significant. To determine predictors of serum hepcidin isoform concentrations, univariate regression analysis was first undertaken, and variables where $p = > 0.1$ were excluded from further multivariate regression analysis. To correct for skewness, concentrations were log transformed prior to univariate and multivariate regression analysis.

4.3 Results

Samples were obtained from 71 patients with CKD not undergoing haemodialysis, and an additional 34 patients pre- and post-haemodialysis; demographics are shown in Table 4.2 and Table 4.3. Unfortunately, samples were not available from individuals with stage 5 CKD, not requiring haemodialysis.

Patients with CKD and those on haemodialysis were significantly older than the control group. The ratio of males to females was comparable for the control group and those patients with CKD requiring haemodialysis. However, there were more males with CKD not requiring haemodialysis as compared to the control group and those patients requiring haemodialysis. Patients with CKD not requiring dialysis, and those requiring dialysis had significantly higher serum CRP and ferritin, and significantly lower serum iron, TIBC, and TSAT, as compared to the control group. Some 57 % of CKD patient's not requiring dialysis had a raised CRP, as compared to 74 % of patients on haemodialysis. The proportion of patients with a raised CRP did not differ markedly between stages of CKD (stage 1: 63 %, stage 2: 50 %, stage 3: 44 %, and stage 4: 69 %).

Table 4.2. Clinical and demographic data for controls and patients with CKD not undergoing haemodialysis. Data shown as median (range), hepcidin-22 was not detected in any sample (LLoQ 1 µg/L). Insufficient numbers to report median (range) hepcidin-24 concentrations for individual CKD stage.

	Controls (N = 41)	Stage 1 (N = 8)	Stage 2 (N = 14)	Stage 3 (N = 27)	Stage 4 (N = 22)	All CKD (N = 71)	Controls vs. all CKD p-value
Male (%)	44	38	57	74	50	59	-
Age (years)	37 (24–68)	41 (20–75)	49 (33–74)	61 (32–84)	65 (28–90)	57 (20–90)	< 0.001
Haemoglobin (g/dL)	-	135 (101–145)	134 (109–168)	135 (73–154)	117 (81–165)	127 (73–168)	-
Serum creatinine (µmol/L)	-	62 (54–79)	87 (76–121)	143 (83–200)	227 (159–300)	143 (54–300)	-
eGFR (mL/min/1.73 m^2)	-	105 (98–145)	68 (60–89)	41 (31–59)	24 (17–28)	41 (12–145)	-
CRP (mg/dL)	3.0 (< 2.0–10.0)	7.7 (2.1–19.2)	5.4 (2.8–7.9)	5.4 (2.3–123.2)	6.5 (2.2–28.1)	6.1 (2.1–123.2)	< 0.05
Ferritin (µg/L)	66 (20–348)	60 (24–279)	125 (20–379)	174 (14–1495)	127 (13–953)	128 (13–1495)	< 0.001
Iron (µmol/L)	16.4 (7.3–31.7)	13.6 (4.3–25.7)	14.2 (8.3–22.3)	11.9 (3.9–43)	11.1 (4.5–26.7)	12.2 (3.9–43)	< 0.001
TIBC (µmol/L)	57 (46–70)	56 (47–79)	56 (46–76)	52 (35–70)	51 (29–74)	53 (29–79)	< 0.001
TSAT (%)	29 (12–63)	27 (7–43)	26 (17–37)	22 (11–84)	21 (9–79)	23 (7–84)	< 0.01
Hepcidin-20 (µg/L)	ND	2 (1–2)	2 (1–6)	5 (1–24)	8 (1–31)	5 (1–31)[1]	-
Hepcidin-24 (µg/L)	ND	ND	ND	ND	ND	3 (2–14)[2]	-
Hepcidin-25 (µg/L)	8 (1–31)	6 (3–42)	14 (2–58)	23 (5–69)	16 (4–134)	14 (2–134)[3]	< 0.01
'Total' hepcidin (µg/L)	8 (1–31)	5 (1–42)	5 (1–58)	8 (1–69)	10 (1–134)	8 (1–134)	< 0.01

Key ND = not detected (LLoQ 1 µg/L) 1. Hepcidin-20 detected in 73 % of samples, 2. Hepcidin-24 detected in 20 % of samples, 3. Hepcidin-25 detected in 87 % of samples.

Table 4.3. Clinical and demographic data for controls, and samples from patients pre-, and post-haemodialysis. Data shown as median (range).

Insufficient numbers to report median (range) hepcidin-24 concentrations for samples taken post-haemodialysis.

	Demographics and analytical results			Significance testing between data sets (p-value)			
	Controls (N = 41)	Pre-haemodialysis (N = 34)	Post-haemodialysis (N = 34)	Controls vs. pre-HD	Controls vs. post-HD	Pre-HD vs. post-HD	All CKD vs. Pre-HD
Male (%)	44	44	-	-	-	-	-
Age (years)	37 (24–68)	59 (27–87)	-	< 0.001	-	-	0.33
Haemoglobin (g/dL)	-	103 (56–137)	-	-	-	-	< 0.001
CRP (mg/dL)	3.0 (< 2.0–10.0)	10.6 (2.6–124.3)	-	< 0.001	-	-	< 0.05
Ferritin (µg/L)	66 (20–348)	339 (23–3834)	502 (28–5044)	< 0.001	< 0.001	< 0.05	< 0.001
Iron (µmol/L)	16.4 (7.3–31.7)	6.9 (1.0–31.8)	10.9 (4.8–38.5)	< 0.001	< 0.01	< 0.001	< 0.001
TIBC (µmol/L)	57 (46–70)	35 (17–60)	42 (21–57)	< 0.001	< 0.001	< 0.001	< 0.001
TSAT (%)	29 (12–63)	19 (4–84)	25 (8–93)	< 0.001	0.39	< 0.05	0.35
Hepcidin-20 (µg/L)	ND	18 (5–129)[1]	10 (5–73)	-	-	< 0.05	< 0.001
Hepcidin-22 (µg/L)	ND	19 (9–32)[2]	15 (10–19)	-	-	-	-
Hepcidin-24 (µg/L)	ND	9 (5–51)[3]	ND	-	-	-	< 0.05
Hepcidin-25 (µg/L)	8 (1–31)	54 (11–234)[4]	35 (4–143)	< 0.001	< 0.001	< 0.05	< 0.001
'Total' hepcidin (µg/L)	8 (1–31)	78 (11–387)	49 (4–228)	< 0.001	< 0.001	< 0.05	< 0.001

Key 1. Hepcidin-20 detected in 71 % of samples, 2. Hepcidin-22 detected in 9 % of samples, 3. Hepcidin-24 detected in 26 % of samples, 4. Hepcidin-25 detected in 88 % of samples. 'Total' hepcidin = sum of all hepcidin isoforms.

4.3.1 Hepcidin in CKD patients not requiring haemodialysis

Hepcidin-25 (median, range) was significantly raised in samples from patients with CKD not requiring haemodialysis (14, 2–134 µg/L) as compared to the control group (8, 1–31 µg/L, $p = < 0.01$), although some 61 % of samples had a hepcidin-25 concentration that was within the range of concentrations measured in the control group (Figure 4-1). Overall, hepcidin-25 was positively correlated with ferritin ($r = 0.55$, $p = < 0.001$), and negatively correlated with TIBC ($r = -0.45$, $p = < 0.01$), haemoglobin ($r = -0.34$, $p = < 0.01$), and eGFR ($r = -0.33$, $p = < 0.01$), Figure 4-2, and Figure 4-3. There was no statistically significant correlation of hepcidin-25 to iron, TSAT or CRP (Table 4.4).

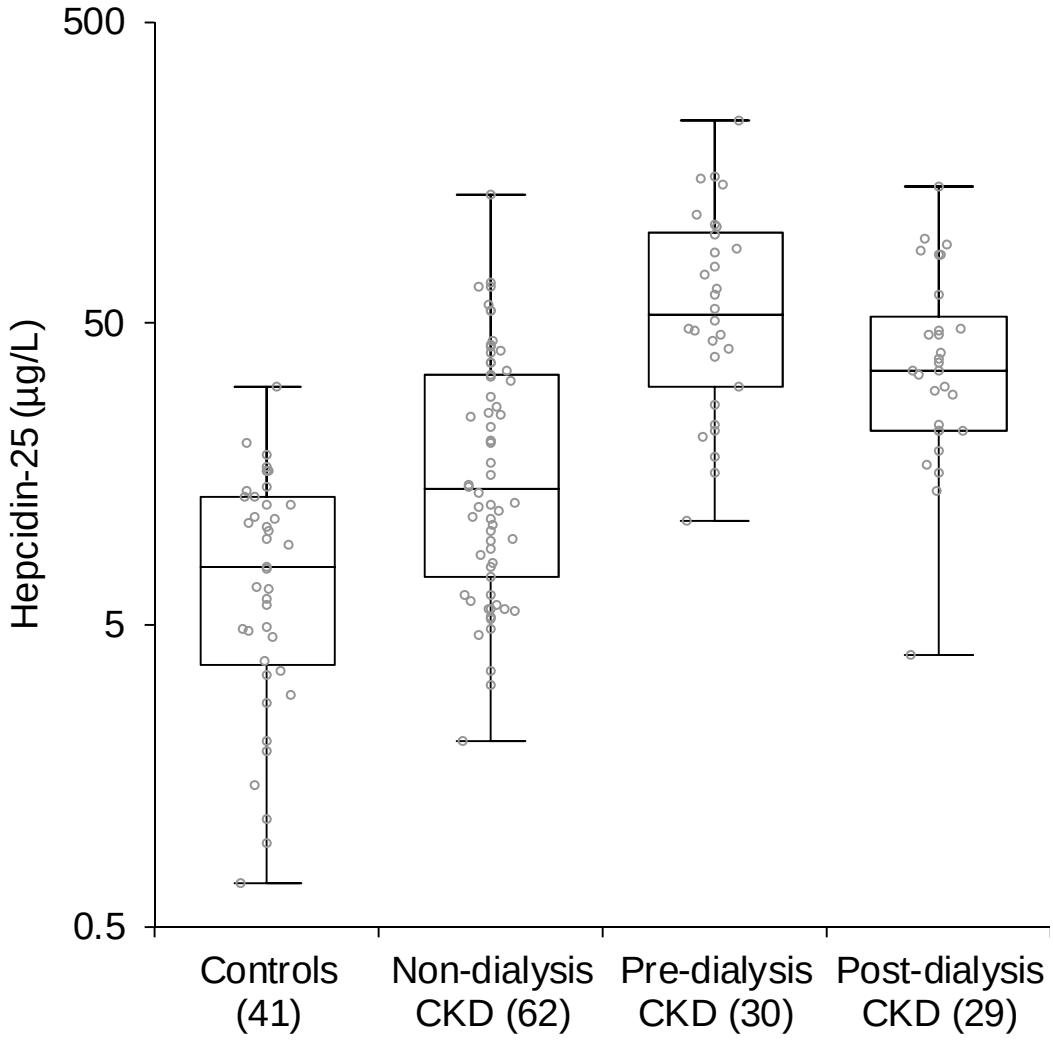


Figure 4-1. Concentrations of hepcidin-25 in controls, CKD patients not undergoing haemodialysis and in samples taken pre, post-haemodialysis (hepcidin-25 on logarithmic scale, and excludes samples < 1 µg/L, figure in parenthesis = number of samples).

Table 4.4. Correlation of hepcidin isoforms with haematological and biochemical indices in serum samples from patients with CKD not requiring haemodialysis. Hepcidin-22 not detected (LLoQ 1 µg/L) in any sample.

	Parameter						
	Haemoglobin	CRP	Ferritin	Iron	TIBC	TSAT	eGFR
Hepcidin-20							
r	-0.40	0.24	0.44	-0.03	-0.58	0.12	-0.60
p	< 0.01	0.17	< 0.001	0.83	< 0.001	0.40	< 0.001
Hepcidin-24							
r	-0.49	0.50	0.51	-0.62	-0.75	-0.35	-0.04
p	0.07	0.17	0.06	< 0.05	< 0.01	0.22	0.89
Hepcidin-25							
r	-0.34	0.24	0.55	-0.21	-0.45	-0.10	-0.33
p	< 0.01	0.15	< 0.001	0.10	< 0.01	0.44	< 0.01

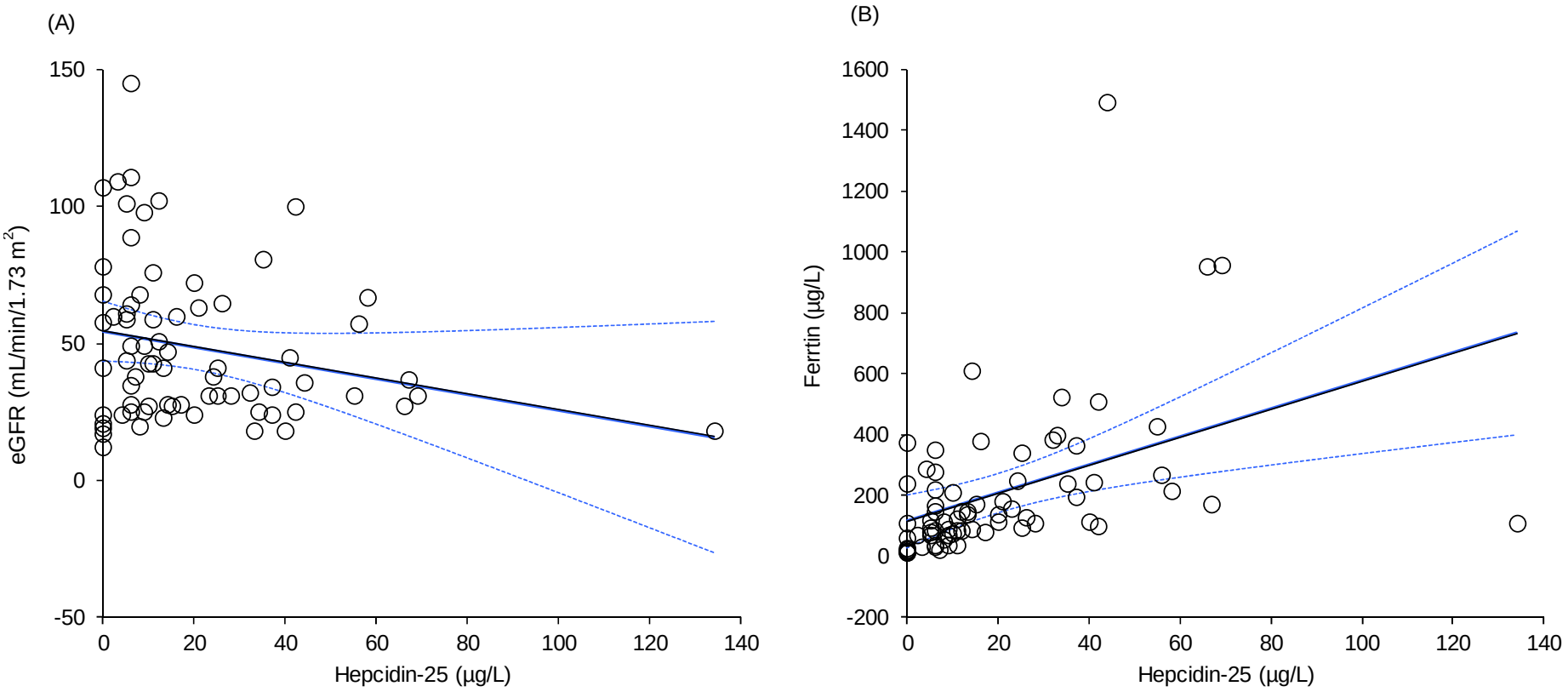


Figure 4-2. Scatterplots of hepcidin-25 with (A) eGFR, and (B) ferritin in patients with CKD not requiring dialysis. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

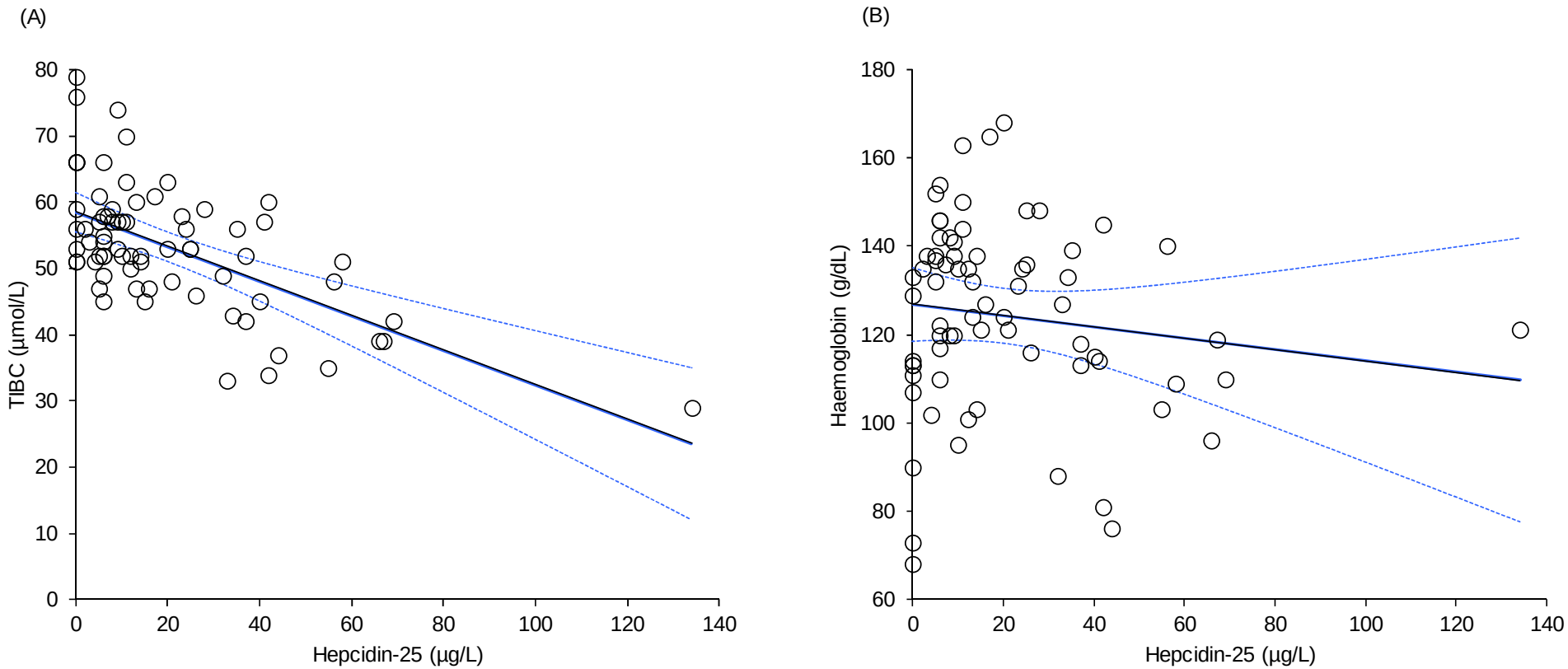


Figure 4-3. Scatterplots of hepcidin-25 with (A) TIBC, and (B) haemoglobin in patients with CKD not requiring dialysis. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

Multivariate regression analysis, including the predictors eGFR, ferritin, TIBC, and haemoglobin only explained 30 % of the variation in serum hepcidin-25 concentrations; TIBC ($p = < 0.05$) was the only significant predictor of hepcidin-25 concentrations.

Hepcidin-20 was similarly correlated to haemoglobin ($r = -0.40$, $p = < 0.01$), ferritin ($r = 0.44$, $p = < 0.001$), TIBC ($r = -0.58$, $p = < 0.001$), and eGFR ($r = -0.60$, $p = < 0.001$) (Figure 4-4, and Figure 4-5, Table 4.4). Multivariate regression analysis, including the predictors haemoglobin, ferritin, TIBC and eGFR, showed TIBC ($p = < 0.001$), and eGFR ($p < 0.01$), to be significant predictors of hepcidin-20. Hepcidin-24 was negatively correlated with iron ($r = -0.62$, $p = < 0.05$), and TIBC ($r = -0.75$, $p = < 0.01$), but was not correlated to haemoglobin, CRP, ferritin, TSAT or eGFR (Figure 4-6, Table 4.4).

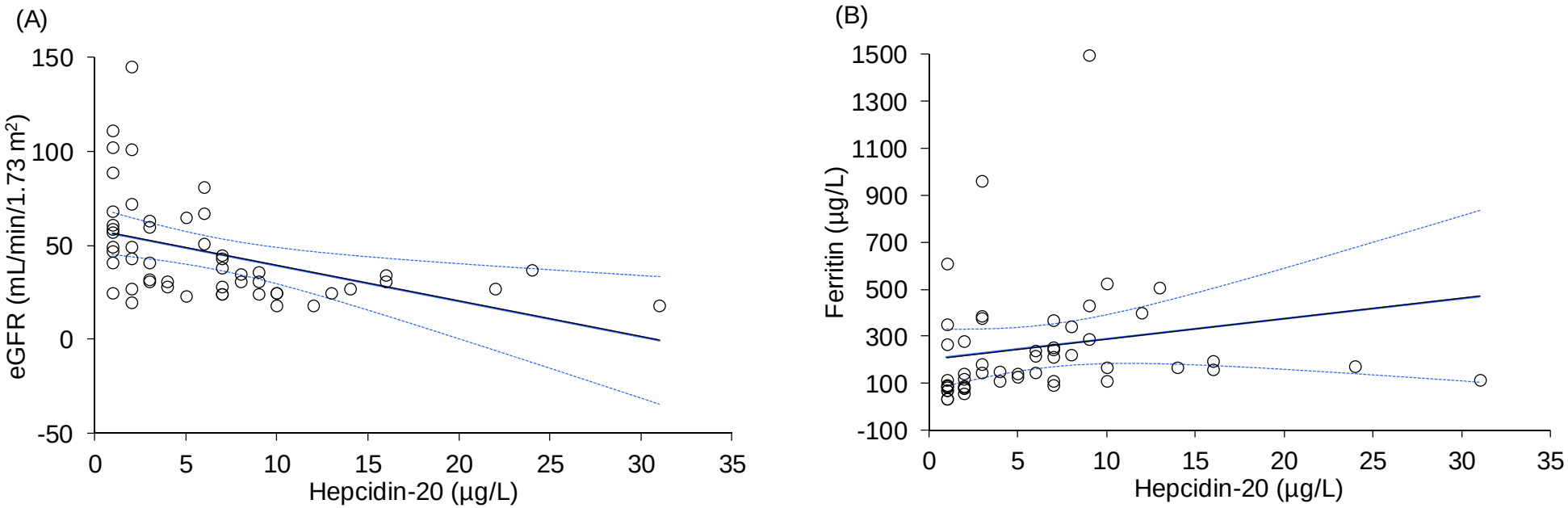


Figure 4-4. Scatterplots of hepcidin-20 with (A) eGFR, and (B) ferritin in patients with CKD not requiring dialysis. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

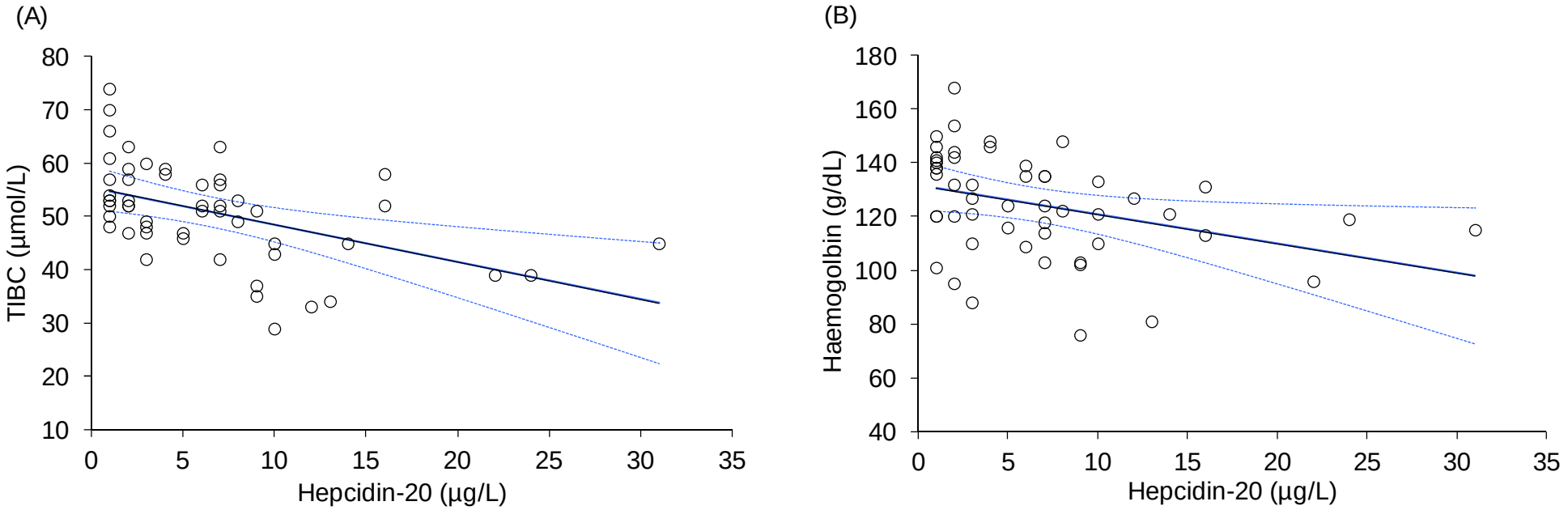


Figure 4-5. Scatterplots of hepcidin-20 with (A) TIBC, and (B) haemoglobin in patients with CKD not requiring dialysis. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

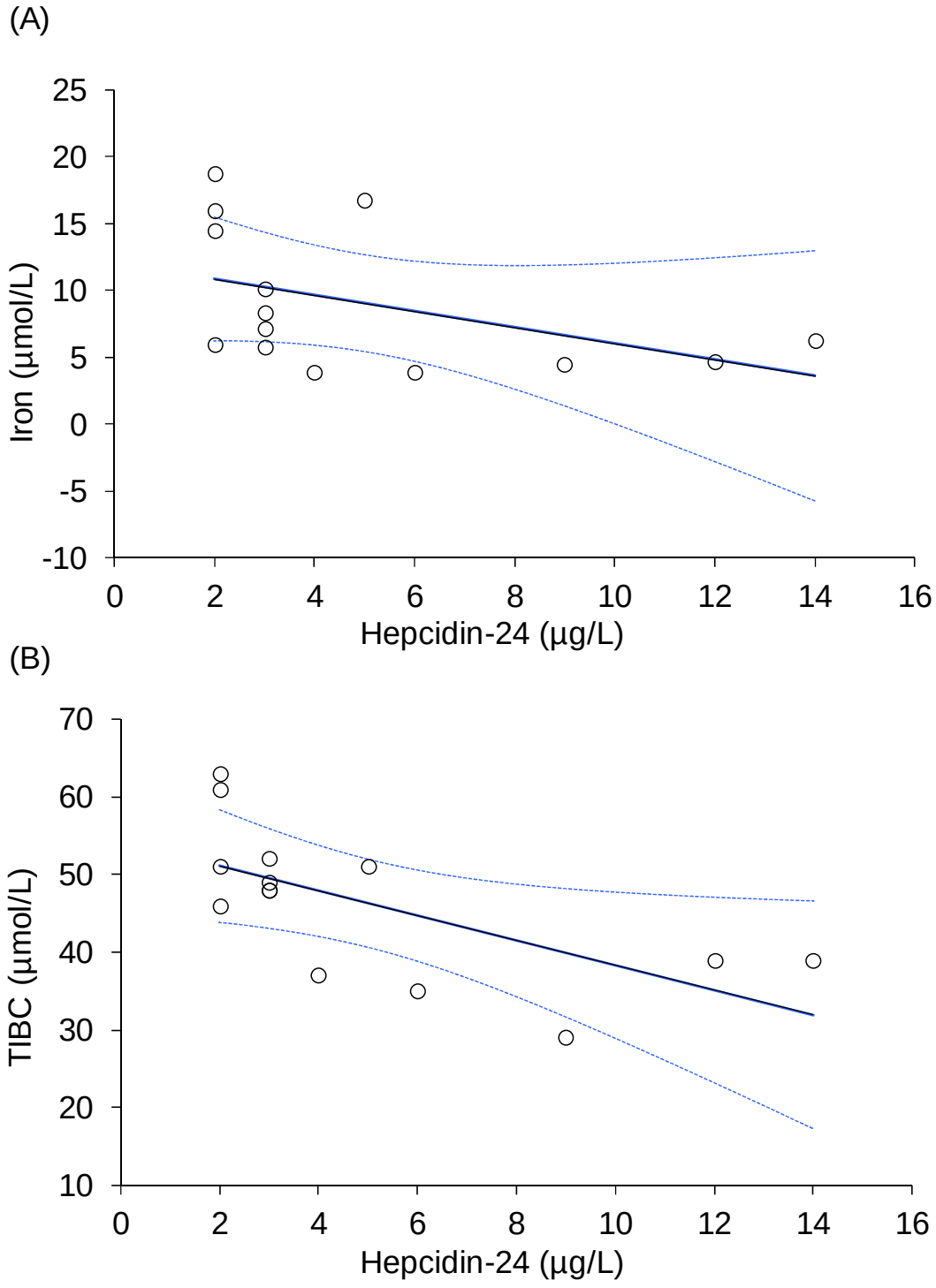


Figure 4-6. Scatterplots of hepcidin-24 with (A) iron, and (B) TIBC. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

In all samples from patients with CKD not requiring dialysis, hepcidin-25 was the most prevalent isoform of hepcidin detected (Figure 4-7), and was the only hepcidin isoform detected in 14 % of samples (median 6, range 2–42 µg/L).

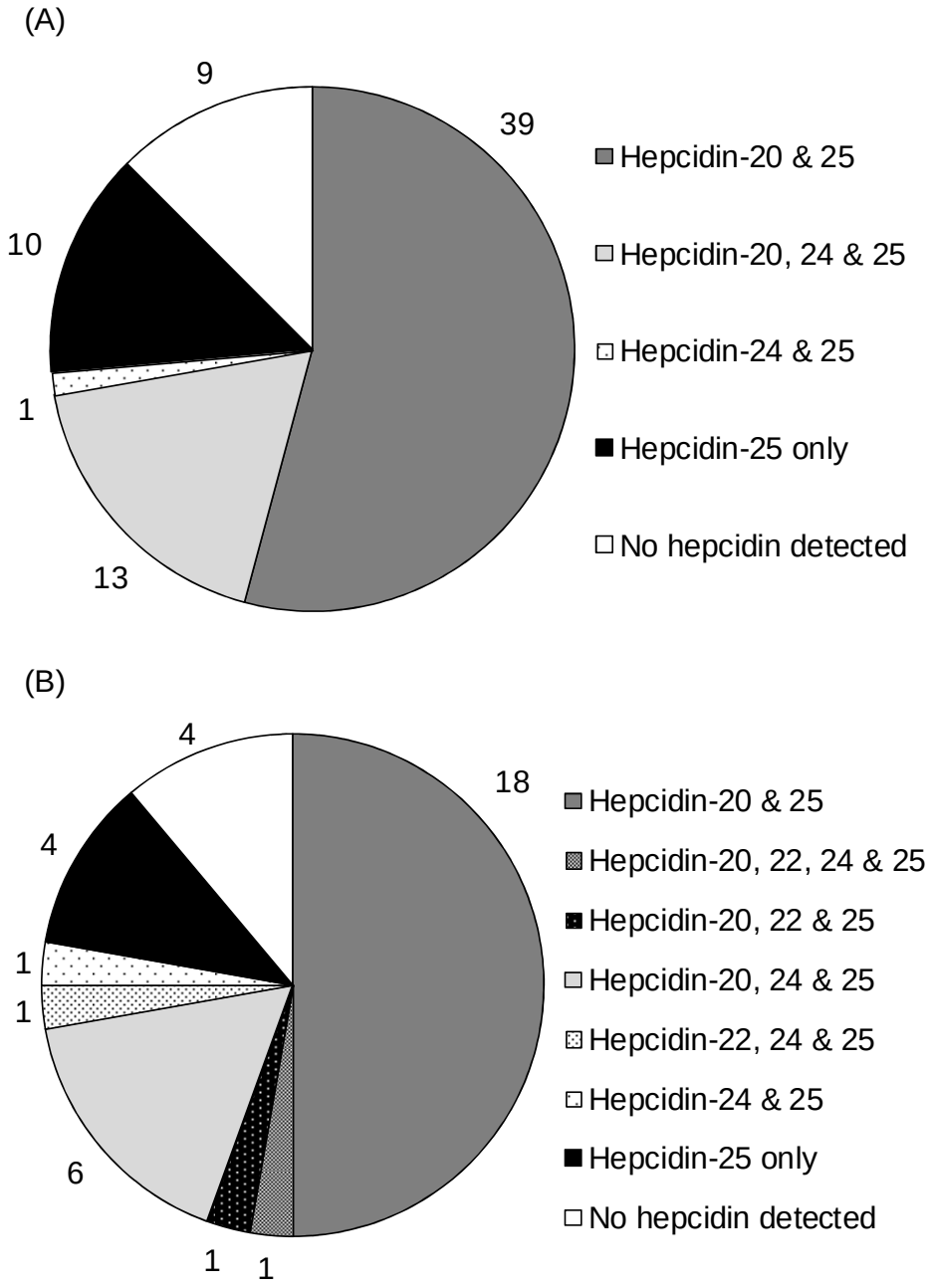


Figure 4-7. Prevalence of hepcidin isoforms in samples from (A) patients with CKD not requiring dialysis (N = 72), and (B) samples from haemodialysis patients taken pre-dialysis (N = 34). Figure next to segment denotes number of samples.

Hepcidin-25, was not detected in 10 samples, and of these, 8 had a low haemoglobin (median 109, 68–114 g/dL), 6 had a low ferritin concentration (median 19, range 13–27 µg/L), and 9 had a low TSAT (median 17, range 5–26 %), suggesting that these patients had absolute iron-deficiency that would explain an undetectable hepcidin-25 concentration.

Besides hepcidin-25, hepcidin-20 was the most prevalent hepcidin isoform (72 % of samples), and its contribution to the 'total' hepcidin concentration (i.e. the sum of hepcidin-20, -22, -24, and -25 isoforms where measured) increased with declining kidney function (Figure 4-8 and Figure 4-9).

Hepcidin-24 was detected in 19 % of samples, but the proportion of hepcidin-24 to the total hepcidin concentration measured was consistent across all samples irrespective of kidney function. Hepcidin-22 was not detected in any sample, and in no sample was hepcidin-20, -22, or -24 detected in the absence of hepcidin-25.

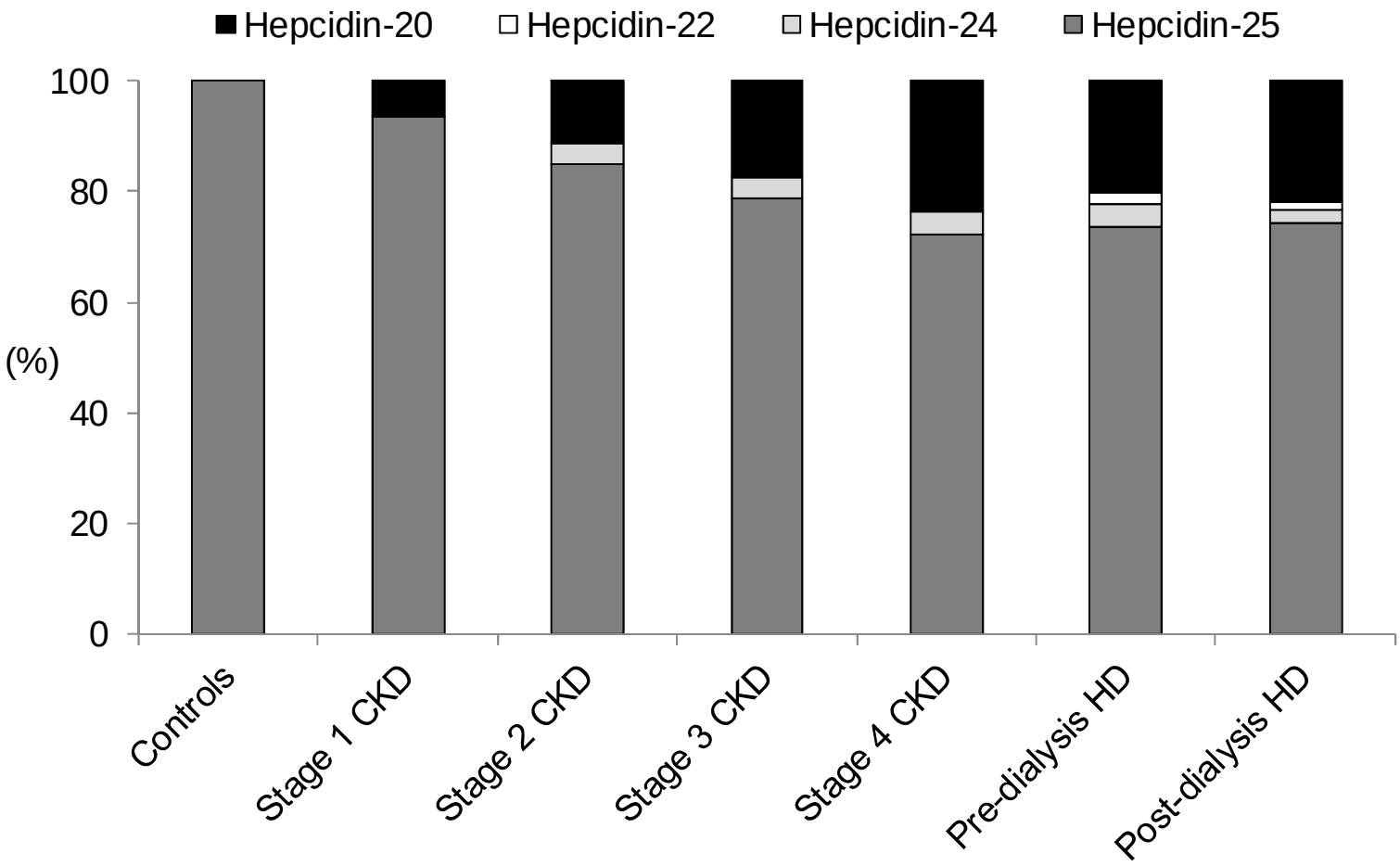


Figure 4-8. Percentage contribution of each hepcidin isoform to the total hepcidin concentration measured.

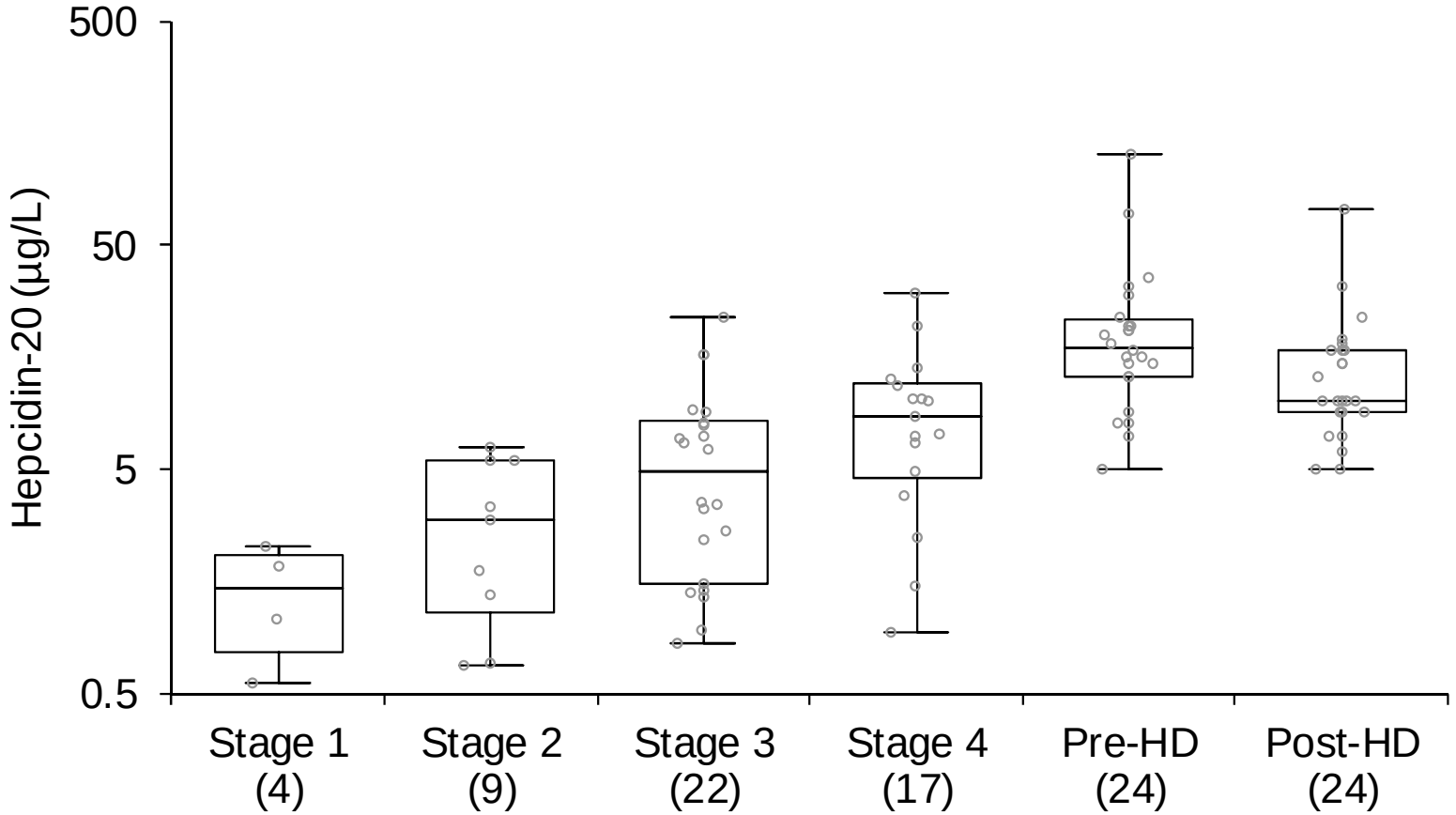


Figure 4-9. Concentrations of hepcidin-20 in CKD patients not undergoing haemodialysis by stage of disease and in samples taken pre-, and post-haemodialysis (hepcidin-20 on logarithmic scale, and excludes samples < 1 µg/L, figure in parenthesis = number of samples).

4.3.2 Hepcidin in CKD patients requiring haemodialysis

Hepcidin-25 was significantly raised in samples taken pre-dialysis (median 54, range 11–234 µg/L) as compared to both the control group (median 8, range 1–31 µg/L) and patients with CKD not requiring haemodialysis (median 8, range 1–134 µg/L). Although, 28 % of samples had a measurable hepcidin-25 concentration that was within the range found in healthy controls. Hepcidin-25 was only correlated to TIBC (r = -0.38, p = < 0.05), with no significant correlation to CRP, ferritin, iron, or TSAT (Table 4.5).

Table 4.5. Correlation of hepcidin isoforms with biochemical tests in serum samples taken pre-haemodialysis.

	CRP	Ferritin	Iron	TIBC	TSAT
Hepcidin-20					
r	0.03	0.14	-0.11	-0.35	-0.16
p	0.92	0.50	0.60	0.09	0.44
Hepcidin-24					
r	0.51	0.79	-0.37	-0.34	-0.47
p	0.19	< 0.05	0.33	0.36	0.20
Hepcidin-25					
r	0.24	0.30 (0.42)[1]	-0.27	-0.38	-0.28
p	0.28	0.11 (< 0.05)[1]	0.16	< 0.05	0.14

Insufficient number of hepcidin-22 concentrations (9, 19, and 32 µg/L) to calculate correlation.

Key. 1. Excludes outlier (hepcidin-25 = 16 µg/L, ferritin = 3,834 µg/L).

However, excluding an outlier (hepcidin = 16 µg/L, ferritin = 3,834 µg/L) improved the correlation of hepcidin-25 to ferritin (r = 0.42, p = < 0.05). Multivariate regression analysis, including the predictors CRP, iron, and TIBC, only explained 31 % of the variation in serum hepcidin-25 concentrations, and none were significant predictors of hepcidin-25. Hepcidin-20 was not correlated to any variable investigated, and hepcidin-24, was only correlated with ferritin (r = 0.79, p = < 0.05, Table 4.5).

Hepcidin-25 was the most prevalent isoform of hepcidin detected in samples taken pre-dialysis (Figure 4-7). Hepcidin-25, was not detected in 4 samples, and in these, 2 had a low haemoglobin concentration (65 and 96 g/dL), 1 had a ferritin concentration that was low (23 µg/L), and 3 had a low TSAT (median 16, range 6–16 %). As with samples from patients with CKD not requiring haemodialysis, besides hepcidin-25, hepcidin-20 was the most prevalent isoform (76 % of samples). Hepcidin-22 and 24 were detected in 8 and 25 % of samples respectively. Hepcidin-25 was the only hepcidin detected in 11 % of samples (median 19, range 11–152 µg/L), and in no sample were hepcidin-20, -22 or -24 detected in the absence of hepcidin-25.

Following haemodialysis, median (range), hepcidin-20, -22, -24, and -25 concentrations had declined by some 38 (1–55), 44 (41–47), 34 (33–35), and 35 (6–71) %, respectively (Figure 4-10). In all patients, hepcidin-20, -22, -24, and -25 had declined after dialysis, except in one patient where hepcidin-20 had increased from 7 µg/L (pre-dialysis) to 17 µg/L (post-dialysis). In one patient, hepcidin-22 was below the LLoQ post-dialysis (pre-dialysis: 9 µg/L), and in a further 7 patients, hepcidin-24 was below the LLoQ, post-dialysis (pre-dialysis: 5, 6, 7, 8, 9, 10, and 16 µg/L).

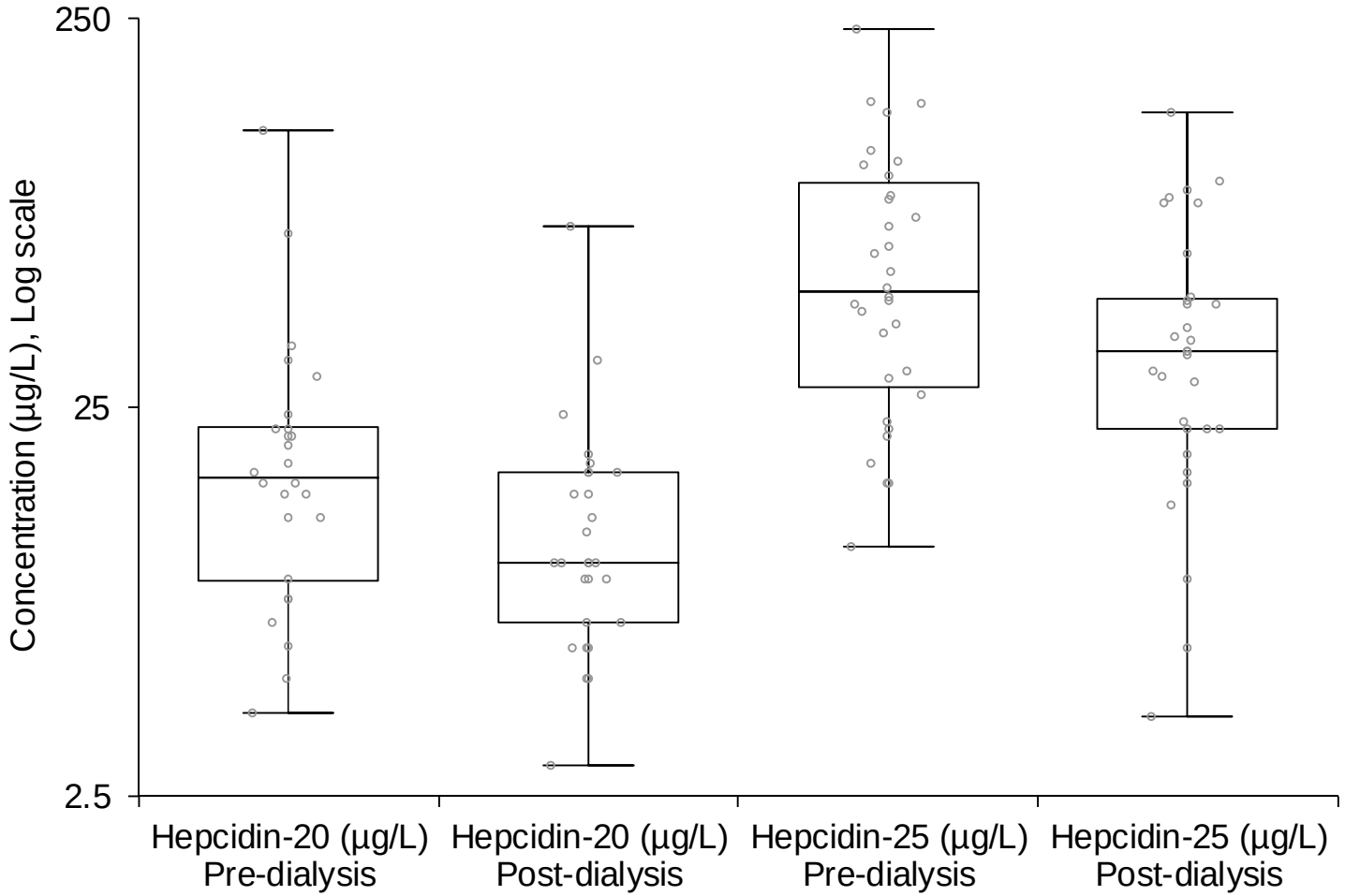


Figure 4-10. Concentrations of hepcidin-20 (N = 24), and hepcidin-25 (N = 30) in samples taken pre-, and post-haemodialysis.

4.3.3 Hepcidin isoform correlations in CKD patients

Correlations of hepcidin-25 to hepcidin-20, and -24 are shown in Figure 4-11. In patients with CKD not requiring haemodialysis, serum hepcidin-25 was strongly correlated to hepcidin-24 (N = 14, r = 0.82, p = < 0.05), and to a lesser extent with hepcidin-20 (N = 52, r = 0.52, p = < 0.05). Hepcidin-24 was also moderately correlated with hepcidin-20 (N = 13, r = 0.64, p = < 0.05). In samples taken pre-dialysis, there was a likewise strong correlation of hepcidin-25 with hepcidin-24 (N = 9, r = 0.85, p = < 0.05), and again to a lesser extent with hepcidin-20 (N = 26, r = 0.70, p = < 0.05). There was no statistically significant correlation of hepcidin-24 with hepcidin-20 (N = 7, r = 0.34, p = 0.45). There were an insufficient number of samples (N = 3) to investigate correlations with hepcidin-22.

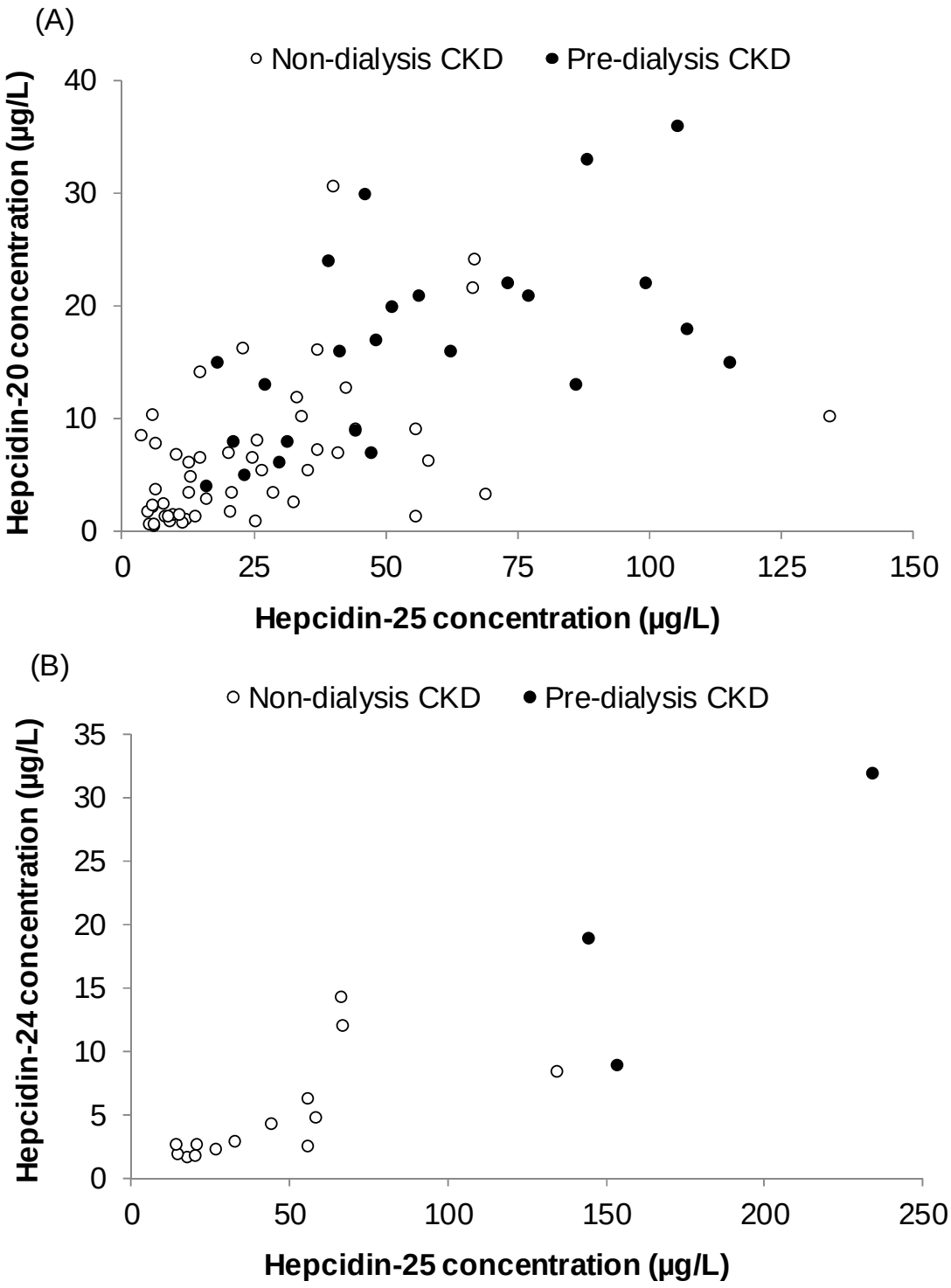


Figure 4-11. Correlation of serum hepcidin-25 with (A) hepcidin-20, and (B) hepcidin-24. Two samples excluded from graph A for clarity (hepcidin-20: 70 & 129 µg/L, and hepcidin-25: 234 & 144 µg/L, respectively).

4.4 Discussion

In the work undertaken here, all commercially available hepcidin isoforms have been measured in individuals with CKD not undergoing haemodialysis, and in samples from individuals undergoing haemodialysis both before and after the dialysis session. Hepcidin-25, and hepcidin-20 were found to be the most prevalent forms of hepcidin present in samples, and the concentrations of hepcidin-24 present have been ascertained, which have not been reported to date.

4.4.1 Hepcidin isoforms in CKD not requiring haemodialysis

A number of studies have reported concentrations of hepcidin-25 in patients with CKD (both not requiring and requiring haemodialysis) using either mass spectrometry (Weiss *et al.,* 2009; Peters *et al.,* 2010; Tessitore *et al.,* 2010; Kuragano *et al.,* 2010; Rumjon *et al.,* 2012; Uehata *et al.,* 2012; Pelusi *et al.,* 2013; van der Weerd *et al.,* 2012; Chand *et al.,* 2015; Addo *et al.,* 2016) or immunochemical assays (Malyszko *et al.,* 2006; Ford *et al.,* 2010; Troutt *et al.,* 2013; Xu *et al.,* 2011; Zaritsky *et al.,* 2011; Mercadel L *et al.,* 2014; Mogadam *et al.,* 2015; Taheri *et al.,* 2015; Wagner *et al.,* 2015).

Concentrations of hepcidin-25 measured here in patients with CKD not undergoing haemodialysis are broadly comparable to those reported by other investigators (Peters *et al.,* 2010; Rumjon *et al.,* 2012; Uehata *et al.,* 2012), except in one report where the concentrations of hepcidin-25 measured using an ELISA were 4-fold greater than those reported here (Mogadam *et al.,* 2015). It is likely that the cause of this disparity is due to the different methodology used to quantify hepcidin-25, and a lack of assay standardisation as reported in round robins (Kroot *et al.,* 2009; Kroot *et al.,* 2012; van der Vorm *et al.,* 2016).

There are conflicting results as to whether patients with CKD not undergoing dialysis have raised hepcidin-25 as compared to healthy controls. In the samples measured here, hepcidin-25 was significantly raised as compared to healthy controls, although there was much overlap, with over half of these samples having a hepcidin-25 concentration that was within the range measured in healthy controls. One published study reported there to be no significant difference in hepcidin-25 concentrations between healthy controls and in patients with CKD not undergoing haemodialysis when using an MS based method (Peters *et al.,* 2010). In contrast, other investigators have reported hepcidin-25 to be significantly raised (2-, 3 to 6-, and 20-fold

respectively) in non-dialysing CKD patients as compared to healthy controls (Malyszko *et al.,* 2006; Uehata *et al.,* 2012; Troutt *et al.,* 2013). It should be borne in mind though that the study that reported hepcidin-25 to be 20-fold higher in CKD patients compared to controls used an immunoassay for hepcidin measurement, and it is likely that other hepcidin isoforms cross-reacted with the assay (Troutt *et al.,* 2013). In this work, *N*-truncated isoforms of hepcidin-25, predominantly hepcidin-20, were found to be raised in patients with CKD. It could therefore be possible that the immunoassay method used by Troutt *et al.* (2013) could have reported falsely elevated 'hepcidin-25' concentrations, although this is unlikely to be the sole cause of the disparity in this case since, in the samples here, the median hepcidin-20 concentration (the most prevalent hepcidin isoform, besides hepcidin-25) measured was only 36 % of the measured hepcidin-25 concentration.

In one study using mass spectrometry (Peters *et al.,* 2010), hepcidin-20 and -22 were present in 81 % and 34 % of samples respectively, and the concentrations of hepcidin-20 measured (median: 3.6, range: 1.2–12.8 µg/L) were broadly comparable to those reported here (median: 5, range: 1–31 µg/L), although the ratio of hepcidin-25 to -20 reported was higher (4.6) than in the samples in this study (2.8). Only three other studies have reported concentrations of hepcidin isoforms (hepcidin-20 and hepcidin-22 alongside hepcidin-25) in patients with CKD (Tessitore *et al.,* 2010; van der Weerd *et al.,* 2012; Addo *et al.,* 2016). The method used in all four of these reports, however, used hepcidin-24 as an internal standard, thereby compromising accurate hepcidin-20, -22, and -25 determination. To date no studies have reported concentrations of hepcidin-24 in patients with CKD.

The *in-vitro* stability studies undertaken in Chapter 3 identified that when hepcidin-25 was added to stripped human serum, neither hepcidin-20, -22, or -24 were detected. There was also no degradation to a smaller hepcidin isoform when either hepcidin-20, -22, or -24 were added to separate portions of stripped human serum. Similarly, there was no evident increase in hepcidin-20, -22, or -24 as hepcidin-25 concentrations decreased when clinical samples were stored at ambient room temperature or at 2–8 °C. Also, when each individual isoform was added to separate portions of stripped human serum, concentrations of all hepcidin isoforms declined at comparable rates. This data needs to be interpreted with caution, especially when trying to extrapolate to the *in-vivo* formation of hepcidin isoforms in patients with CKD. However, what the stability studies undertaken and the data from patients here do suggest, is that *N*-truncated isoforms of hepcidin-25 are unlikely to be formed *in-vivo* and are not artefacts of

sample preparation or storage. It is likely that in individuals with CKD, based on the correlations between the isoforms, hepcidin-25 is not metabolised sequentially (i.e. hepcidin-25, to hepcidin-24, to hepcidin-22 etc), but instead hepcidin-25 could form hepcidin-22, and then hepcidin-20, or even directly from hepcidin-25 to hepcidin-20. Furthermore, hepcidin-25 may not undergo any metabolism before being eliminated from the body or it may just be degraded to hepcidin-24 for example, and then eliminated, without being degraded to hepcidin-20, or -22 . There are still conflicting results as to whether hepcidin-25 is largely protein bound, and whether being bound impedes degradation. For example, it may be possible that only non-protein bound hepcidin-25 is degraded, therefore in conditions where the plasma protein concentrations change, so would the degree of binding, and therefore the available 'non-protein' bound hepcidin-25 available for degradation.

It is not clear if concentrations of the *N*-truncated isoforms of hepcidin-25 are raised as a result of reduced eGFR, or simply because these patients have raised hepcidin-25 concentrations and there is then increased analyte available for degradation to smaller isoforms. In the samples analysed here from patients with CKD not requiring dialysis, hepcidin-20 was more strongly correlated to eGFR than hepcidin-25 was to eGFR, suggesting that hepcidin-20 concentrations are more dependent on eGFR than those of hepcidin-25. As hepcidin-20 concentrations increase with the severity of kidney dysfunction (Figure 4-9), hepcidin-20 may be almost entirely renally cleared, and therefore it may be a suitable biomarker for kidney function, as has been suggested in a recent study (Addo *et al.,* 2015). However, further work of a larger cohort of patients under controlled conditions is required to investigate the relationship between eGFR and isoforms of hepcidin, and their concentrations in other disease states (i.e. sepsis or ACD). In the meantime, however, hepcidin-20 is unlikely to be a suitable biomarker alone for renal function, as hepcidin-20 (and other isoforms of hepcidin-25) maybe increased in other conditions besides CKD (e.g. sepsis, Laarakkers *et al.,* 2013). *In-vitro* studies have shown hepcidin-20 to have little, if any, activity at the FP-1 receptor (Laarakkers *et al.,* 2013), but given the relatively high concentrations measured here to those of hepcidin-25, any biological function identified in the future may be clinically significant in patients with CKD.

4.4.1.1 Predictors of hepcidin concentrations

In patients with CKD not undergoing dialysis, TIBC was the only significant predictor of hepcidin-25 concentration in multivariate regression analysis. Although there were moderate

correlations with ferritin, haemoglobin, and eGFR in univariate analysis, reviewing the scatterplots of these data, however, do not suggest a particularly convincing relationship. These findings are comparable to reported studies that have found both ferritin and TIBC to be correlated with hepcidin-25 (Ashby *et al.,* 2009; Peters *et al.,* 2010; Uehata *et al.,* 2012; Chand *et al.,* 2013; Troutt *et al.,* 2013), and those identified in healthy volunteers reported in Chapter 3. These correlations may not be surprising as a raised ferritin concentration would suggest adequate iron stores (in the absence of inflammation), and a reduced need for dietary iron uptake, and therefore raised serum hepcidin-25. Conversely, TIBC is raised in iron-deficiency, and lower when iron stores are normal to raised. Patients with iron deficiency in the absence of any confounders (e.g. inflammation), therefore, would be expected to have a low ferritin, low hepcidin-25 concentration, and a raised TIBC; patients with adequate iron stores would be expected to have a raised ferritin and hepcidin-25 concentration, but a low TIBC.

There are conflicting results as to whether hepcidin-25 is correlated with eGFR. In one study using an ELISA (Zaritsky *et al.,* 2009), 'total hepcidin' was correlated with eGFR. However, in studies using mass-spectrometry (Peters *et al.,* 2010; Uehata *et al.,* 2012) this correlation was not present. In the samples analysed here, hepcidin-25 was negatively, though weakly, correlated with eGFR in univariate analysis, but eGFR was found not to be a significant predictor of hepcidin-25 concentrations in multivariate regression. The relationship between hepcidin-25 and eGFR has clearly not been resolved; however, since there is no conclusive evidence, it is likely that concentrations of circulating hepcidin-25 are not predominantly dependent on glomerular filtration. It is possible that the raised hepcidin-25 concentrations present in some patients with CKD may be due to the up-regulation of synthesis from inflammatory cytokines that are present from the inflammation which is common in these patients, as opposed to reduced renal clearance. Certainly, over half of the patients with CKD not requiring dialysis had a raised CRP (median 6.1, range 2.1–123.2 mg/dL), as compared to only 4 % (N = 2) of samples in the control group, who only had marginally raised CRP concentrations (6.3, and 9.7 mg/L).

Hepcidin-25 was found to be significantly correlated to haemoglobin in univariate analysis but not in multivariate analysis. As with eGFR, there are conflicting data as to whether hepcidin-25 is correlated with haemoglobin; some studies have reported a correlation between haemoglobin and hepcidin-25 concentrations (Maruyama *et al.,* 2012; van der Putten *et al.,* 2010; Uehata *et al.,* 2012), whereas others have reported no such correlation (Peters *et al.,*

2010). The reason of this disparity could be due to differences in the underlying cause of anaemia (e.g. IDA or ACD) or inflammatory status of the populations studied, or because of different sample sizes.

Hepcidin-20 was correlated with haemoglobin, ferritin, TIBC and eGFR; these results were similar to hepcidin-25 which would be expected given that there was a correlation, albeit weak, ($r = 0.52$, $p = < 0.05$) between hepcidin-25 and -20. The correlation of hepcidin-24 with the measured biochemical and haematological indices was not possible to interpret fully as there were too few samples containing this analyte ($N = 14$), although there was a significant and strong correlation of hepcidin-24 with TIBC, again which may not be surprising as there was good agreement between hepcidin-24 and hepcidin-25 concentrations ($r = 0.82$, $p = < 0.05$).

4.4.2 Effect of haemodialysis on hepcidin concentrations

Concentrations of hepcidin-25 were significantly raised in CKD patients requiring dialysis as compared to both healthy controls and in those patients with CKD not undergoing dialysis, consistent with previous reports (Peters *et al.,* 2010; Ashby *et al.,* 2009). Furthermore, in univariate regression there was a significant correlation of hepcidin-25 with TIBC and ferritin when an outlier was excluded, and again these findings are comparable to those reported by other investigators (Peters *et al.,* 2010; Costa *et al.,* 2009; Kato *et al.,* 2008; Kuranagano, *et al.,* 2010; Tomosugi *et al.,* 2006; Valenti *et al.,* 2009; van der Weerd *et al.,* 2012). However, in multivariate regression analysis, none were significant predictors of serum hepcidin-25.

Following dialysis, serum hepcidin-25 concentrations declined by some 35 %, although there was much variation between individuals. A slightly lower clearance of 10–15 % (Peters *et al.,* 2010), and 27 % (Kuragano *et al.,* 2010) has previously been reported, whereas other investigators have reported higher clearances of 45 % (Zaritsky *et al.,* 2010), and 51 % (Campostrini *et al.,* 2010).

Haemodialysis was also found to significantly reduce the concentrations of hepcidin-20, -22, and -24, which may not be surprising given their very similar molecular weights to hepcidin-25, and that they also have an amphipathic structure, which is known to make these analytes prone to adsorption to surfaces that they come into contact with. It is not clear whether hepcidin isoforms are cleared during the dialysis procedure directly, or whether they adhere to the dialysis membrane. It is likely, however, that both contribute to the decline, since hepcidin-25

has been identified in both the ultrafiltrate and on the dialysis membrane post-haemodialysis (Malyszko *et al.,* 2009; Peters *et al.,* 2010).

The finding that haemodialysis significantly reduces concentrations of hepcidin-25 would suggest that immediately post-haemodialysis is the most appropriate time for administration of oral iron to maximise absorption; however, the median hepcidin-25 concentration in samples post-dialysis was still some 6-fold greater than those measured in samples from healthy volunteers, and may therefore still be too high to allow maximal iron absorption.

4.4.3 Utility of hepcidin measurement in patients with CKD

In most patients with CKD, measurement of hepcidin-25 will provide little additional information as to an individual's irons stores, or the amount of iron in circulation, when compared to traditional markers of iron status such as ferritin, TIBC, and TSAT. This is because hepcidin-25 concentrations are not determined purely by renal function, but also affected by other factors including inflammation. Since hepcidin-25 is an acute phase response protein it is raised in response to inflammation as is ferritin and, in the absence of inflammation, patients that have low iron stores will have a low plasma ferritin as well as a likely low hepcidin-25 concentration. Furthermore, measurement of hepcidin-25 as a predictor of haemoglobin response to intravenous iron in patients with CKD on haemodialysis performed no better than traditional markers of irons status such as ferritin or TSAT in one study (Tessitore *et al*., 2010).

However, hepcidin-25 measurement does provide an almost direct indication as to whether iron would be absorbed if given orally, something which no current marker of iron status can provide. Therefore, it could be helpful in deciding whether oral or IV iron would be most successful in the treatment of anaemia in CKD, although there have been no clinical trials undertaken to prove this theory. Of course, hepcidin-25 measurement would only give an indication as to an individual's ability to absorb iron at the time of when the sample is taken, and its usefulness would therefore be based upon the ease at which hepcidin-25 could be measured, and the speed at which the results are available. It is interesting that in several samples from patients with CKD, undetectable concentrations of hepcidin-25 were found; this would suggest that in these patients, were oral iron supplementation to be given, there could be maximal absorption and utilisation. But again, there is no clinical data to support this hypothesis.

4.4.4 Limitations

As regards to limitations of the work undertaken here, samples were from a naturalistic group of patients diagnosed with CKD. Some of these individuals may be being treated with ESAs, IV or oral iron, or be receiving no treatment for anaemia if present. Furthermore, the time of sampling was not kept constant, and hepcidin-25 has been shown to have a diurnal rhythm. However, the work here does give an indication as to the concentrations of hepcidin isoforms to be expected in this group of patients in a realistic, routine clinical setting.

4.5 Conclusion

Hepcidin-20 was found to increase with a decrease in kidney function, and only in samples from individuals with CKD undergoing haemodialysis were all hepcidin isoforms present. Furthermore, all hepcidin isoforms in serum were found to be significantly reduced by the haemodialysis procedure, a finding which is novel to the work undertaken here. Currently there is no conclusive evidence as to the value of measuring hepcidin-25 in individuals with CKD, although it may be useful in deciding whether to administer oral or IV iron. Due to the proven presence of hepcidin isoforms as renal function declines, any future studies investigating hepcidin-25 concentrations in CKD patients should use a very selective (i.e. mass-spectrometry) assay to ensure accurate quantitation of hepcidin-25, or if an immunoassay is used, at the very least, the cross-reactivity of these isoforms should be assessed.

Chapter 5 Hepcidin in haemochromatosis, anaemias, and sepsis

5.1 Introduction

Both inappropriately low and raised hepcidin-25 concentrations have been implicated in disorders of iron deficiency and excess (Kroot *et al*., 2011). As hepcidin-25 directly controls the presence of FP-1 (Nemeth *et al.*, 2004), and therefore the absorption and cellular release of iron, it is hoped that measurement of hepcidin-25 may be useful for the clinical management of iron disorders. Furthermore, even though *N*-truncated isoforms of hepcidin-25 have been shown to have little or no activity at the FP-1 receptor, they may still have an as yet unidentified physiological role, and may prove useful in interpreting hepcidin-25 concentrations.

In this chapter, concentrations of hepcidin isoforms will be investigated in samples from individuals with iron overload [e.g. haemochromatosis, congenital dyserythropoietic anaemia (CDA II), non-alcoholic fatty liver disease], and iron deficiency (e.g. IDA, ACD). Hepcidin isoforms will also be investigated in samples from individuals known to have sepsis, which is known to induce hepcidin-25 synthesis through inflammatory cytokine activity. Furthermore, the correlation between individual hepcidin isoforms will be explored to identify if this changes between disease states.

The aims of this chapter are as follows:

- Investigate the concentration of hepcidin isoforms in individuals with IDA, ACD, ID, and Sickle Cell Anaemia.
- Investigate the concentration of hepcidin isoforms in individuals with hereditary haemochromatosis of varying genotypes, non-alcoholic fatty liver disease, and non-HFE iron overload.
- Investigate the concentration of hepcidin isoforms in individuals with sepsis.

5.2 Materials and Methods

Iron-deficient anaemia (IDA) was defined as a low haemoglobin (normal range, males; 130–165, females; 115–155 g/dL), a low TSAT [normal range (males & females); 20–50 %], a raised TIBC [normal range (males & females); 50–72 µmol/L] and a low ferritin (normal range, males; 20–300, females; 20–200 µg/L). Anaemia of Chronic Disease (ACD) was defined as a low haemoglobin, a raised ferritin, a low TSAT, and a low TIBC. Iron-deficiency without anaemia was identified as a low ferritin concentration but a normal haemoglobin concentration.

5.2.1 Patient samples

Patients with hereditary haemochromatosis and non-haemochromatosis related iron overload attended a liver outpatient's clinic at King's College Hospital, London for routine monitoring. Patients with sickle cell anaemia, and CDA attended a haematology outpatient's clinic, also at King's College Hospital again for routine monitoring. During the clinics a serum (BD Vacutainer SST II Advance) sample was taken for routine biochemical tests (including ferritin, CRP, TIBC, iron, creatinine) as part of their on-going care. Biochemical tests were undertaken within the Blood Sciences Laboratory of King's College Hospital, in accordance with local procedures (Chapter 3.2.7). The soluble transferrin receptor (STfR, LLoQ: 3 nmol/L) was measured by ELISA (R&D Systems, Minneapolis, USA). The manufacturer suggested target range for STfR is (males and females) is 8.7–28.1 mol/L.

After completion of all biochemical tests excess serum was stored into 1.5 mL protein LoBind tubes at -20 ºC, for up to six months until analysis for hepcidin isoforms. All samples were taken as a part of routine care, excess sample was fully anonymised, and all linkages with the sample to the patient removed, therefore review by a research ethics committee was not required, in accordance with Royal College of Pathologists, and Human Tissue Act Guidelines (Marks, 2012, Human Tissue Authority, 2017). Time taken between collection of the sample and storage for hepcidin measurement was approximately 6 hours, during this time the sample was stored at 2–8 ºC.

Samples obtained from healthy volunteers that were used to establish a reference range for hepcidin isoforms (Chapter 3.3.4) were used as controls for comparison purposes.

5.2.2 Analytical methods

Hepcidin isoforms were measured in singlicate, with a set of calibration solutions and IQCs at the beginning and end of each assay, and an IQC every batch of 10 samples using the LC-HR-MS method previously described (Section 3.2). Biochemical tests were undertaken within the Blood Sciences Laboratory of King's College Hospital, in accordance with local procedures (details Chapter 3.2.5). The soluble transferrin receptor (STfR, LLoQ: 3 nmol/L) was measured by ELISA (R&D Systems, Minneapolis, USA). The manufacturer suggested target range for STfR is (males and females) is 8.7–28.1 mol/L.

5.2.3 Statistical analysis

Statistical analysis was performed using Analyse-it (Analyse-it Software Ltd, Leeds, UK) for Microsoft excel, and SPSS for Windows, version 23.0 (SPSS Incorporated, Chicago, USA). Normality of data distribution was assessed using the Shapiro–Wilk test. Relationships were explored using Spearman's rank correlation (r), simple linear regression analysis (R^2), the Mann–Whitney U (grouped data), or the Wilcoxon signed-rank (matched pairs) test. A p-value < 0.05 was considered statistically significant. Where more than 1 sample from a patient was available, the mean was calculated and used for statistical analysis.

5.3 Results

5.3.1 Anaemia

Demographics of samples analysed are given in Table 5.1, and statistical significance between groups compared to controls is given in Table 5.2. Ten samples were obtained from individuals that had IDA, 10 samples from individuals with ID, 18 samples from individuals with ACD, 12 samples from individuals with SCA (7 HbSS, and 5 HbSC), and 4 samples from an individual with CDA II. A single sample was obtained from each individual, except for 1 individual that had CDA II where 4 samples were obtained. A further 9 samples were received from individuals that had a low TSAT and haemoglobin consistent with IDA, but a ferritin concentration that was within the normal range, CRP was also raised in these 9 samples (Table 5.1).

Hepcidin-25 was significantly lower (p = < 0.0001) in all groups as compared to controls, except in patients with ACD where hepcidin-25 was significantly compared to all other groups (p = < 0.0001) (Figure 5-1). Hepcidin-25 was detected in all samples from individuals

with ACD, but it was only detected in 1 (10 %), 1 (10 %), 1 (25 %), and 4 (33 %) samples from individuals with IDA, ID, CDA II, and SCA, respectively. In samples from individuals with IDA with a ferritin concentration within the normal range, hepcidin-25 was only detected in 3 (33 %) samples, and there was no significant difference in the hepcidin-25 concentration measured in this group and in samples from individuals with IDA but a low ferritin concentration ($p = 0.40$).

Hepcidin isoforms other than hepcidin-25 were only detected in patients with ACD. Hepcidin-20, -22, and -24 were detected in 18 (100 %), 6 (33 %), and 15 (83 %) of these samples respectively (Figure 5-2). In samples from patients with ACD, hepcidin-25 was strongly correlated to hepcidin-24 ($N = 15$, $r = 0.83$, $p = < 0.0001$), and hepcidin-22 ($N = 6$, $r = 0.83$, $p = < 0.05$), but less so to hepcidin-20 ($N = 18$, $r = 0.52$, $p = < 0.05$). Hepcidin-24 was likewise strongly correlated to hepcidin-22 ($N = 6$, $r = 0.83$, $p = < 0.05$), and hepcidin-20 ($N = 15$, $r = 0.87$, $p = < 0.0001$). Hepcidin-22 was not correlated with hepcidin-20 ($N = 6$, $r = 0.54$, $p = 0.27$). With regards to the correlation of hepcidin isoforms with biochemical parameters, hepcidin-25 was only correlated to ferritin ($r = 0.85$, $p = < 0.0001$), as was hepcidin-24 ($r = 0.72$, $p = < 0.05$), and hepcidin-20 ($r = 0.57$, $p = < 0.05$).

Table 5.1. Clinical and demographic data for controls and samples from individuals with IDA, ACD, ID, SCA and CDA II. Data shown as median (range), ND = Not detected (< 1 µg/L).

	Controls (N = 41)	IDA (N = 10)	IDA (normal ferritin, N = 9)	ACD (N = 18)	ID (N = 10)	SCA (N = 12)	CDA II (N = 4)
Male (%)	44	40	100	6	10	67	100
Age (years)	37 (24–68)	41 (22–84)	62 (61–85)	78 (57–88)	52 (20–70)	28 (18–57)	54
Haemoglobin (g/dL)	-	102 (60–116)	93 (68–109)	91 (69–108)	121 (115–142)	103 (72–133)	-
CRP (mg/dL)	3 (< 2–10)	6 (< 2–10)	115 (< 2–168)	116 (9–295)	6 (< 2–24)	6 (< 2–13)	< 2
Ferritin (µg/L)	66 (20–348)	8 (3–19)	72 (32–87)	499 (201–1591)	11 (5–19)	70 (24–292)	56 (25–64)
eGFR (mL/min/1.73 m^2)	-	79 (44–133)	27 (9–24)	57 (13–103)	118 (64–167)	153 (74–236)	-
Iron (µmol/L)	16.4 (7.3–31.7)	2.5 (1.0–6.8)	3.0 (1.4–9.6)	2.4 (1.4–6.7)	6.9 (3.2–14.2)	12.9 (6.4–19.0)	36.8 (30.4–52.7)
TIBC (µmol/L)	57 (46–70)	73 (54–88)	27 (25–83)	30 (21–44)	61 (45–92)	57 (46–71)	59 (56–66)
TSAT (%)	29 (12–63)	3 (1–9)	8 (4–16)	10 (6–17)	12 (4–27)	23 (12–35)	62 (50–92)
Soluble transferrin receptor (nmol/L)	-	-	-	-	-	65.5 (32.5–142.0)	-
Hepcidin-20 (µg/L)	ND	ND	ND	8 (2–72)	ND	ND	ND
Hepcidin-22 (µg/L)	ND	ND	ND	9 (3–88)	ND	ND	ND
Hepcidin-24 (µg/L)	ND	ND	ND	8 (4–45)	ND	ND	ND
Hepcidin-25 (µg/L)	8 (1–31)	1 (<1–2)	1 (1–6)	60 (10–213)	1 (<1–4)	1 (<1–10)	1 (<1–3)

Table 5.2. Statistical significance between groups by variable using the Mann–Whitney U test (statistically significant, p = < 0.05).

	p-value			
	Controls vs. IDA	Controls vs. ACD	Controls vs. ID	Controls vs. SCA
Age	0.49	< 0.0001	0.27	0.05
CRP	0.28	< 0.0001	0.06	0.79
Ferritin	< 0.0001	< 0.0001	< 0.0001	0.52
Iron	< 0.0001	< 0.0001	< 0.0001	< 0.05
TIBC	< 0.001	< 0.0001	0.11	0.59
TSAT	< 0.0001	< 0.0001	< 0.0001	< 0.05
Hepcidin-20	-	-	-	-
Hepcidin-22	-	-	-	-
Hepcidin-24	-	-	-	-
Hepcidin-25	< 0.0001	< 0.0001	< 0.0001	< 0.0001

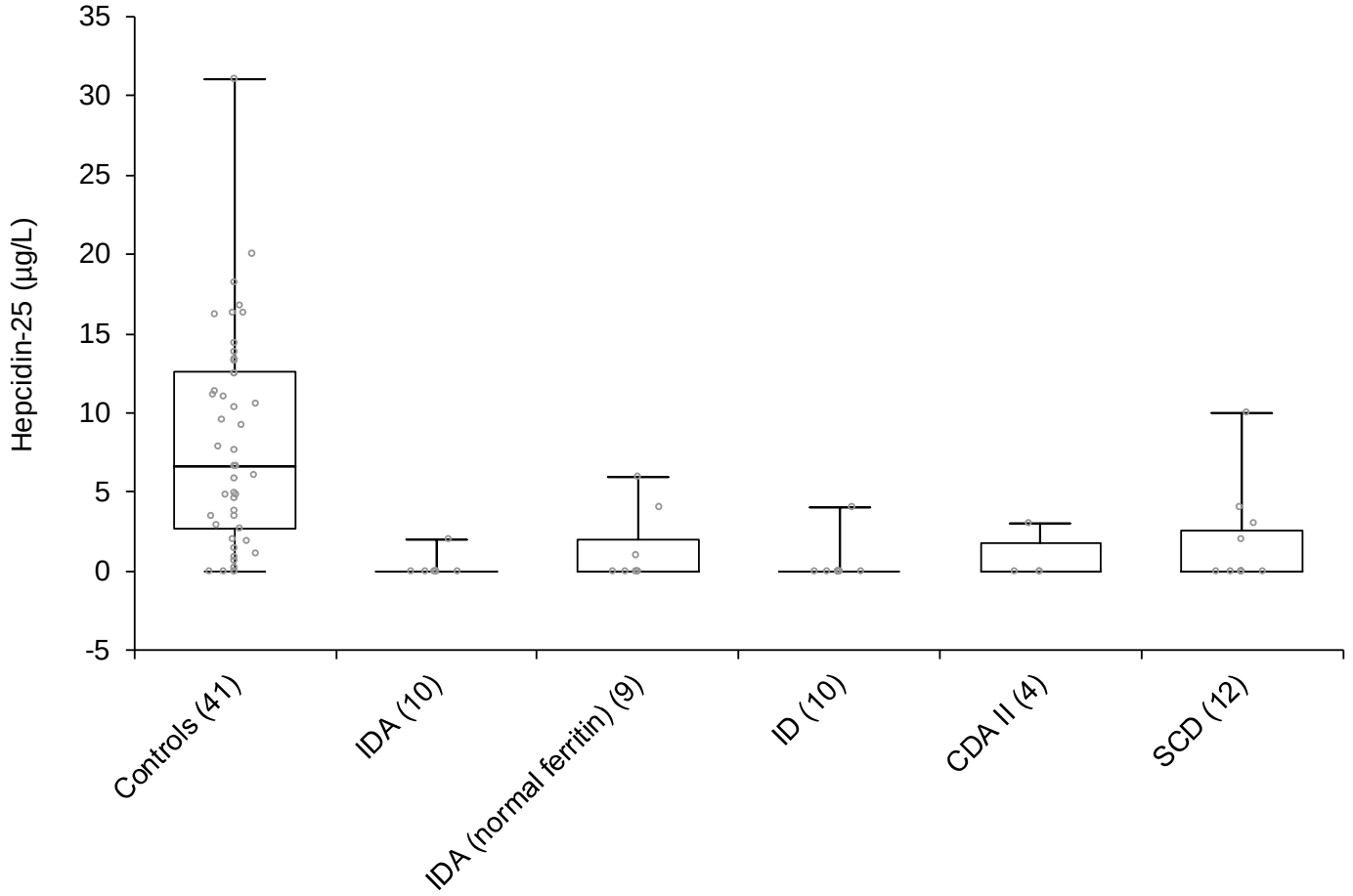


Figure 5-1. Concentrations of hepcidin-25 in controls, and samples from individuals with IDA, IDA (normal ferritin), ID, CDA II, and SCA (number of samples in parentheses).

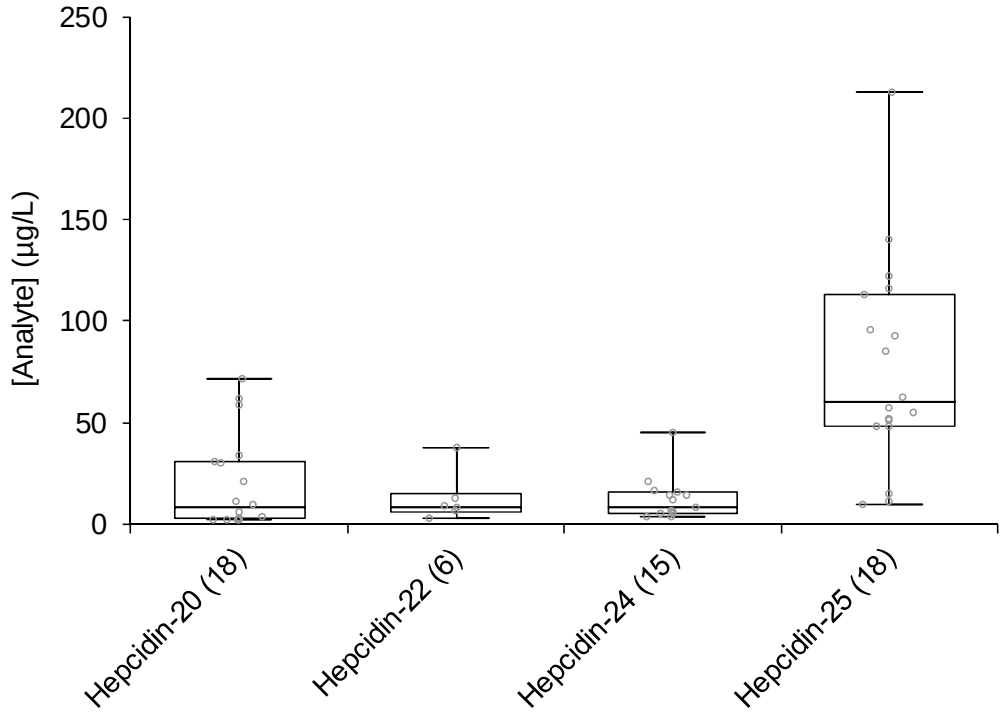


Figure 5-2. Concentrations of hepcidin-20, hepcidin- 22, hepcidin-24, and hepcidin-25 in samples from individuals with ACD (number of samples in parentheses).

5.3.2 HFE-related and non-HFE related iron overload

In total, 86 samples were received from patients with HFE-related iron overload; of these sixty samples were obtained from 33 individuals that were HFE C282Y homozygotes (C282Y/C282Y), 6 samples from 4 individuals that were HFE C282Y heterozygotes (C282Y), 11 samples from 5 individuals that were HFE C282Y compound heterozygotes (C282Y/H63D), and 9 samples from 7 individuals that were HFE H63D heterozygotes (H63D). In addition, 12 samples were received from 10 individuals with NAFLD, and 11 samples from 5 individuals that had non-HFE iron overload. Demographics of samples analysed are given in Table 5.3, and statistical significance between groups and controls is given in Table 5.4.

Hepcidin-25 was not detected in 42 and 17 % of samples from HFE C282Y homozygotes and HFE C282Y heterozygotes, retrospectively. However, hepcidin-25 was detected in all samples from the other groups analysed. Hepcidin-25 was significantly lower in HFE C282Y homozygotes as compared to controls, and was significantly higher in HFE H63D heterozygotes, individuals with non-HFE iron overload, and NAFLD compared to controls (Table

5.4). There was no significant difference in concentrations of hepcidin-25 between controls, HFE C282Y heterozygotes, and HFE C282Y compound heterozygotes (Table 5.4). Hepcidin-25 was significantly higher ($p < 0.05$) in all groups as compared to HFE C282Y homozygotes, and HFE H63D heterozygotes. Individuals with non-HFE iron overload, and NAFLD had significantly higher ($p < 0.05$) hepcidin-25 concentrations as compared to HFE C282Y heterozygotes, and HFE C282Y compound heterozygotes. Hepcidin-25 was significantly higher in individuals with non-HFE iron overload as compared to individuals with NAFLD ($p < 0.05$), but not compared to HFE C282Y compound heterozygotes ($p = 0.76$). There was no significant difference ($p = 0.38$) in hepcidin-25 between HFE C282Y compound heterozygotes and those individuals with NAFLD. Hepcidin-20, -22, or -24 were not detected in any sample.

Table 5.3. Clinical and demographic data for controls and patients with haemochromatosis of different genotypes, NAFLD, and in patients with non-HFE iron overload. Data shown as median (range), ND = Not detected (1 µg/L).

	Controls (N = 41)	C282Y/C282Y (N = 33)	C282Y (N = 4)	C282Y/H63D (N = 15)	H63D (N =7)	Non-HFE iron overload (N = 5)	NAFLD (N = 10)
Male (%)	44	75	30	100	89	82	83
Age (years)	37 (24–68)	51 (33–83)	63 (29–63)	56 (33–84)	61 (36–79)	61 (34–72)	40 (25–67)
CRP (mg/dL)	3 (< 2–10)	-	-	-	-	-	3 (< 2–14)
Ferritin (µg/L)	66 (20–348)	263 (16–1365)	317 (45–2086)	286 (89–670)	478 (24–856)	215 (155–1,233)	149 (63–587)
Iron (µmol/L)	16.4 (7.3–31.7)	28.2 (12.5–44.4)	21.9 (14.5–51.5)	21.4 (16.3–25.6)	24.5 (11.7–46.7)	19.7 (15.6–26.3)	19.4 (11.5–26.0)
TIBC (µmol/L)	57 (46–70)	48 (5–61)	59 (48–60)	60 (55–70)	59 (52–71)	51 (46–60)	65 (56–70)
TSAT (%)	29 (12–63)	59 (21–93)	42 (24–89)	38 (26–47)	42 (21–75)	38 (30–53)	32 (16–37)
Hepcidin-20 (µg/L)	ND	ND	ND	ND	ND	1 (1–2)	ND
Hepcidin-22 (µg/L)	ND	ND	ND	ND	ND	ND	ND
Hepcidin-24 (µg/L)	ND	ND	ND	ND	ND	4 (3–4)	ND
Hepcidin-25 (µg/L)	8 (1–31)	2 (<1–13)	8 (3–22)	4 (2–29)	17 (4–62)	24 (8–46)	11 (5–31)
Ferritin:Hepcidin-25	0.084 (0.001–0.320)	0.023 (< 0.001–0.208)	0.038 (0.005–0.067)	0.022 (0.005–0.063)	0.029 (0.012–0.176)	0.051 (0.037–0.155)	0.058 (0.022–0.208)
%age of samples with detectable hepcidin-25	-	58	83	100	100	100	100

Table 5.4. Statistical significance between groups by variable using the Mann–Whitney U test (statistically significant; p < 0.05).

	p-value					
	Controls vs. C282Y/C282Y	Controls vs. C282Y	Controls vs. C282Y/H63D	Controls vs. H63D	Controls vs. Non-HFE iron overload	Controls vs. NAFLD
Ferritin	< 0.05	0.07	< 0.01	< 0.001	< 0.05	< 0.05
Iron	< 0.0001	0.15	< 0.05	< 0.05	0.09	0.33
TIBC	< 0.0001	0.73	0.39	0.39	0.09	< 0.05
TSAT	< 0.0001	0.19	0.23	0.10	< 0.05	0.94
Hepcidin-25	< 0.0001	0.68	0.76	< 0.05	< 0.0001	< 0.05

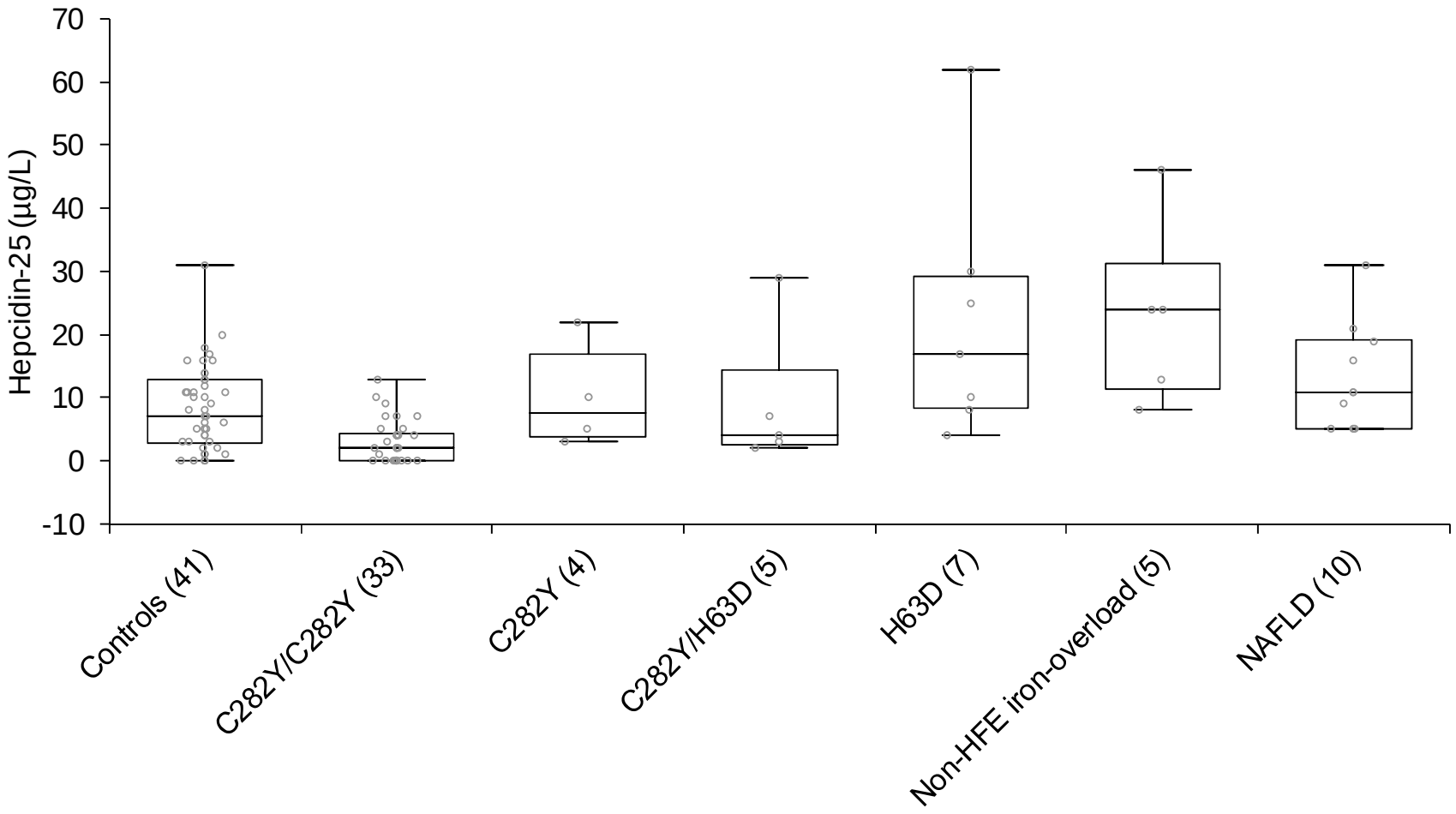


Figure 5-3. Concentrations of hepcidin-25 in controls, in individuals with hereditary haemochromatosis of various genotypes, individuals with non-HFE iron overload, and those with NAFLD (number of samples in parentheses).

5.3.3 Sepsis

Fourteen samples were obtained from individuals with sepsis; demographics are given in Table 5.5. Hepcidin-20, -22, -24, and -25 were detected in 9 (64 %), 8 (57 %), 12 (86), and 14 (100 %) samples. Hepcidin-25 was significantly (p = < 0.0001) raised as compared to controls (Figure 5-4).

Table 5.5. Clinical and demographic data for controls and patients with sepsis. Data shown as median (range).

	Controls (N = 41)	Sepsis (N = 14)	p-value
Male (%)	44	36	-
CRP (mg/dL)	3 (< 2–10)	189 (28–562)	< 0.0001
eGFR (mL/min/1.73 m^2)	-	56 (6–173)	-
Ferritin (µg/L)	66 (20–348)	307 (36–1343)	< 0.0001
Iron (µmol/L)	16.4 (7.3–31.7)	2.0 (1.0–9.7)	< 0.0001
TIBC (µmol/L)	57 (46–70)	37 (23–80)	< 0.0001
TSAT (%)	29 (12–63)	5 (1–13)	< 0.0001
Haemoglobin (g/dL)	-	93 (64–140)	-
Hepcidin-20 (µg/L)	ND	2 (<1–45)	-
Hepcidin-22 (µg/L)	ND	3 (<1–18)	-
Hepcidin-24 (µg/L)	ND	9 (<1–30)	-
Hepcidin-25 (µg/L)	8 (1–31)	92 (11–216)	< 0.0001

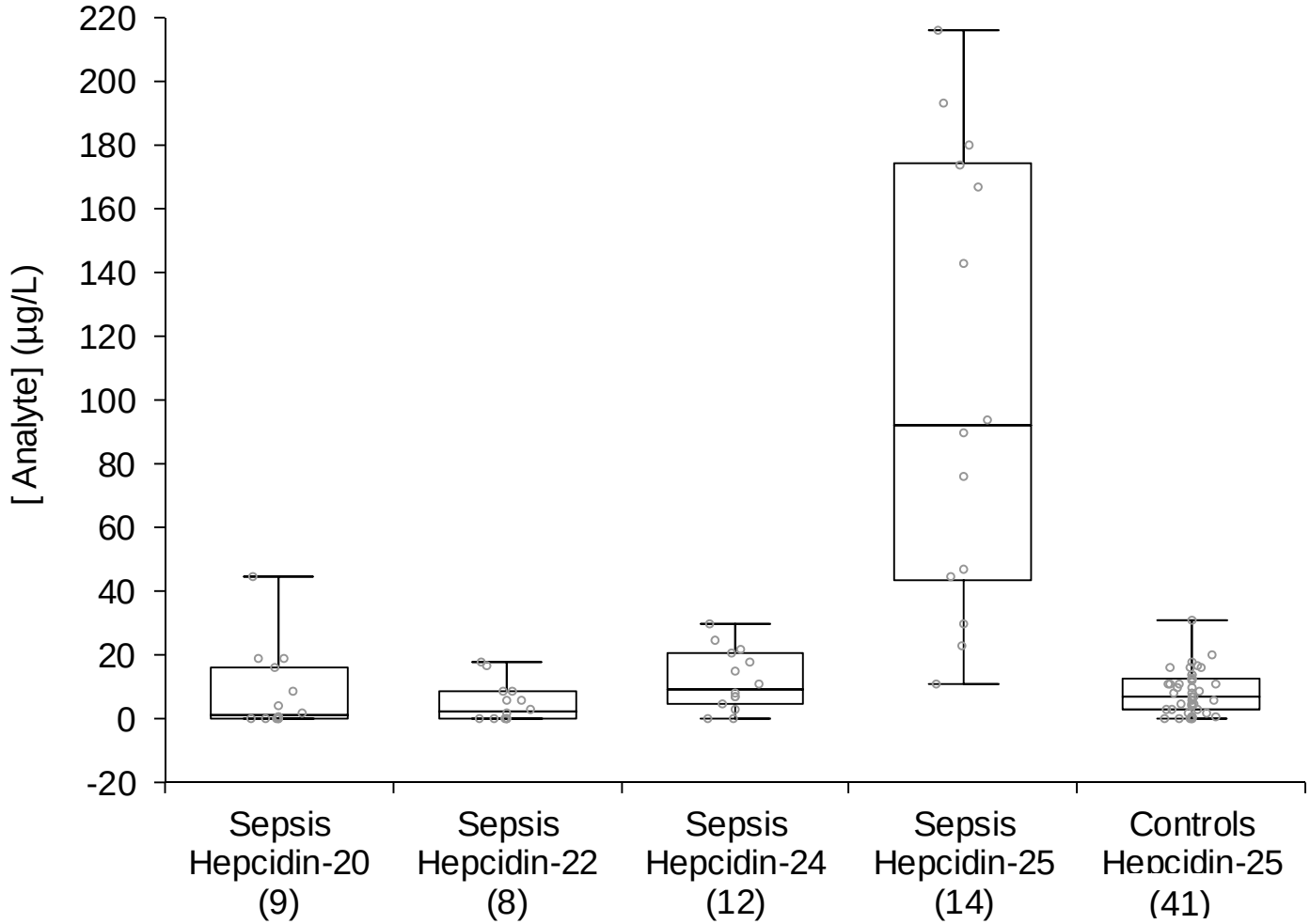


Figure 5-4. Concentrations of hepcidin-20, - 22, -24, and -25 in controls and samples from individuals with sepsis (number of samples in parentheses).

5.3.4 Relationship of hepcidin isoforms between disease states

The correlations of each hepcidin isoform (concentrations from each disease state grouped, not analysed separately) to each other are given in Figure 5-5, Figure 5-6, and Figure 5-7. Hepcidin-25 was strongly correlated with hepcidin-24 (N = 50, r = 0.890, p = < 0.0001), but less so with hepcidin-22 (N = 17, r = 0.62, p = < 0.001), and hepcidin-20 (N = 103, r = 0.62, p = < 0.0001). Hepcidin-24 was also strongly correlated with hepcidin-22 (N = 16, r = 0.85, p = < 0.0001), but less so with hepcidin-20 (N = 44, r = 0.73, p = < 0.0001). Hepcidin-22 was strongly correlated with hepcidin-20 (N = 14, r = 0.87, p = < 0.0001).

There was no statistically significant difference (p = 0.89) in the hepcidin-25:hepcidin-24 ratio between samples from individuals with ACD, sepsis, CKD, and samples from individuals pre-haemodialysis (Figure 5-8). However, samples from individuals with sepsis had a statistically significant raised median hepcidin-25:hepcidin-20 ratio as compared to individuals with CKD (p = < 0.05), and pre-haemodialysis (p = < 0.001). With regards to the ratio of hepcidin-24:hepcidin-20, samples from individuals with ACD had a significantly raised ratio as compared to those samples taken pre-haemodialysis (p = < 0.05). Also samples from individuals with sepsis had a significantly raised hepcidin-24:hepcidin-20 ratio as compared to samples from individuals with CKD (p = < 0.05), and those samples taken from individuals pre-haemodialysis (p = < 0.01).

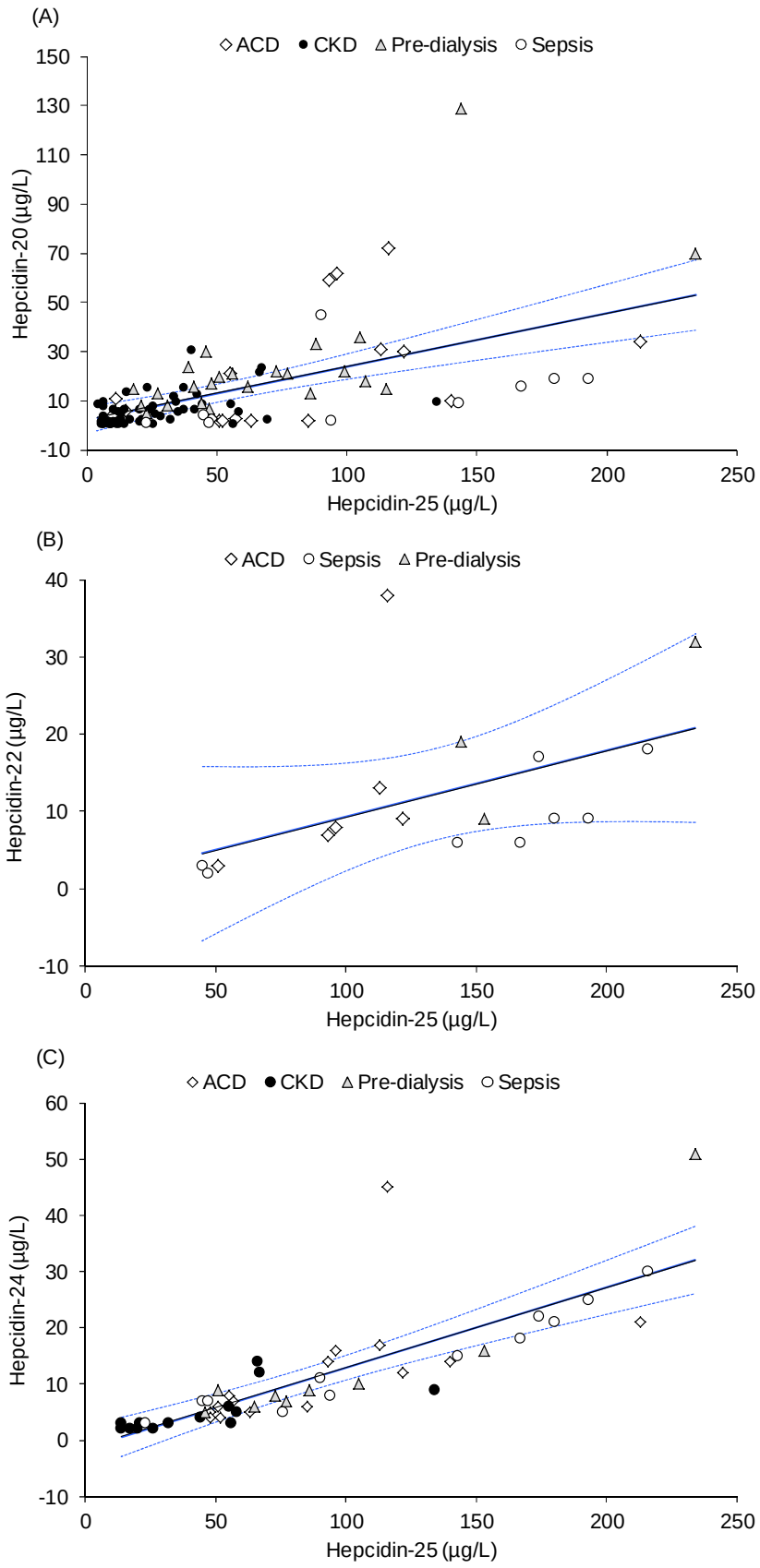


Figure 5-5. Scatterplots of hepcidin-25 with (A) hepcidin-20, (B) hepcidin-22, and (C) hepcidin-24. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

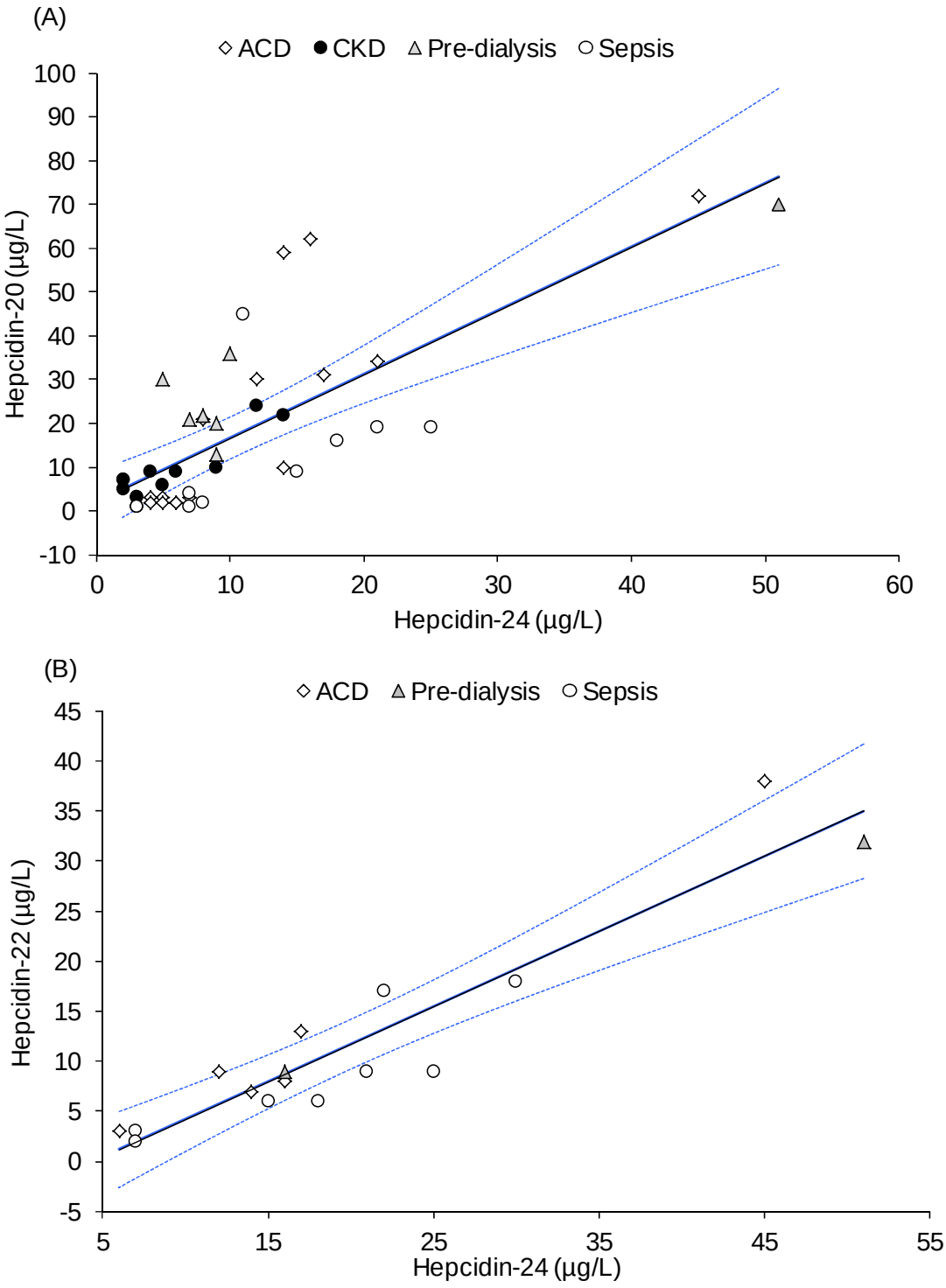


Figure 5-6. Scatterplots of hepcidin-24 with (A) hepcidin-20, and (B) hepcidin-22. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

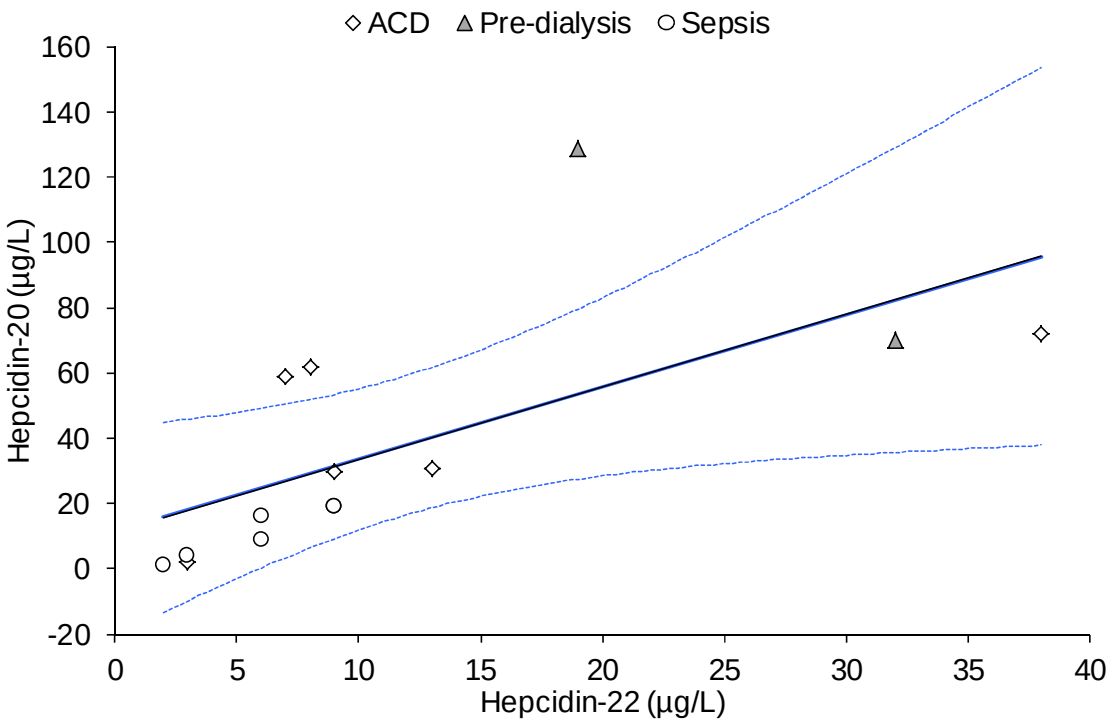


Figure 5-7. Scatterplot of hepcidin-22 with hepcidin-20. Solid line = simple linear regression, dashed lines = 95 % confidence intervals.

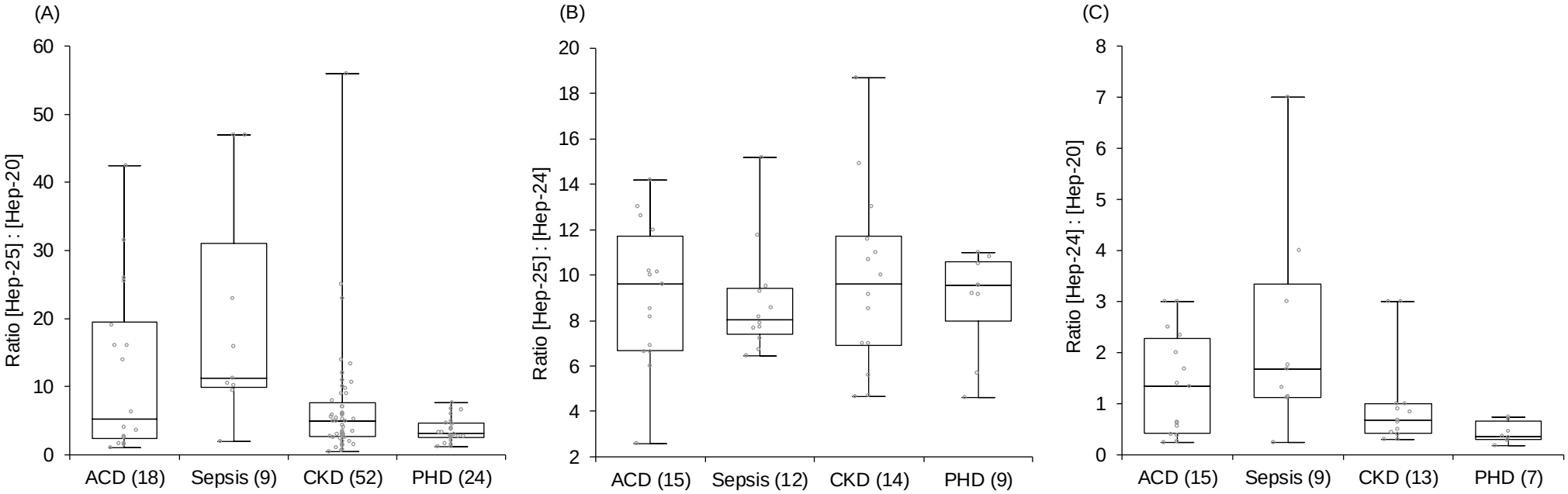


Figure 5-8. Hepcidin isoform ratios by condition (number of samples in parentheses). PHD = pre-haemodialysis.

5.4 Discussion

5.4.1 Hepcidin in iron deficiency anaemia (IDA) and iron deficiency (ID)

Individuals with 'true' IDA (i.e. iron-deficient anaemia, without inflammation or other confounding factors) would be expected to have low hepcidin-25 concentrations to ensure maximal iron absorption. Certainly, this is what is shown in the samples analysed here, where individuals with IDA had significantly lower hepcidin-25 concentrations as compared to controls, and in most cases hepcidin-25 was below the LLoQ of the assay. This finding has also been reported by other investigators (Ganz *et al.,* 2008; Kroot *et al.,* 2010; Bregman *et al.,* 2013).

A sub-set of samples analysed were from individuals with IDA that had a ferritin within the reference range, and a markedly raised CRP. The median hepcidin-25 concentration from this subset was comparable to those with true IDA, but the range of hepcidin-25 concentrations measured were slightly broader. The broader range of hepcidin-25 concentrations measured most likely reflects the up-regulation of hepcidin-25 synthesis due to inflammation and raised circulating cytokines (e.g. IL-6), as identified by a raised CRP. Furthermore, these individuals also had significant renal impairment as indicated by a low eGFR, again possibly contributing to the raised hepcidin-25 as compared to samples from those with 'true' IDA. It is interesting that despite the markedly raised concentrations of CRP present in these individuals, hepcidin-25 was still low and even non-detectable in some samples. Clearly, in these individuals, the down-regulation of hepcidin-25 from reduced iron stores via the BMP-6-HJV-SMAD pathway takes precedence over the stimuli obtained from likely increased concentrations of circulating IL-6 acting on the JAK/STAT pathway. Hepcidin-20, -22, nor -24 were present in any sample from individuals with IDA, which would be expected if they were degradation products of hepcidin-25 since there was little to no hepcidin-25 detected.

In samples from individuals with ID, hepcidin-25 was low, and in many samples, non-detectable. Given that the life span of erythrocytes is approximately 120 days, this finding reflects that hepcidin-25 reacts more rapidly to changes in iron status than haemoglobin content.

5.4.2 Hepcidin in anaemia of chronic disease (ACD)

In ACD, plasma cytokines, particularly IL-6 are elevated, which in-turn induce hepcidin-25 synthesis (Raj, 2009). Therefore, it is not surprising that in samples analysed here from

individuals with ACD, hepcidin-25 was significantly raised (some 6-fold) as compared to the control group and individuals with IDA. This finding has also been reported by other investigators (Kroot *et al.,* 2010; Lasocki *et al.,* 2010; Bergamaschi *et al.,* 2013). Unlike samples from individuals with IDA or ID, *N*-truncated isoforms of hepcidin-25 were present in almost all samples, in-particular hepcidin-20 that was present in every sample. A likely reason for the presence of these isoforms is degradation of hepcidin-25 concentrations *in-vitro*, which is itself present in these samples at raised concentrations due to an increase of synthesis. Of course, ACD has a complicated pathogenesis, and patients with ACD will likely have other co-morbidities that may also cause increases in plasma hepcidin-25 concentrations, such as reduced kidney function.

5.4.3 Hepcidin as a tool to distinguish between IDA and ACD

It has been hoped that measurement of hepcidin-25 may be a useful tool in helping to distinguishing IDA from ACD. In the samples measured here, a low or non-detectable hepcidin-25 concentration would suggest IDA, and a raised hepcidin-25 concentration would suggest ACD. However, there were still samples from individuals with IDA and ACD where hepcidin-25 was within the range measured in controls.

When measured in isolation (without other markers of irons status) hepcidin-25 offers no real benefit compared to other markers of iron status in individuals with IDA, simply because in the samples analysed here all had a low ferritin and TSAT, and a raised TIBC, which are all consistent with IDA, and are well known, and well used criterion for diagnosing IDA (Goodnough *et al.,* 2010). Hepcidin-25 measurement does have potential in the diagnosis of IDA from ACD, when interpreted together with other markers of iron status (e.g. ferritin), and this approach has proved successful in patients with inflammatory bowel disease (Bergamaschi *et al.,* 2013), critical illness (Lasocki *et al.,* 2010), rheumatoid arthritis (van Santen *et al.,* 2011), and cancer related anaemia (Shu *et al.,* 2014). However, there were still samples from individuals with ACD that had a hepcidin-25 that was within the ‘normal’ range. Given hepcidin-20 was detected in all samples from individuals with ACD, it is interesting to consider that if ACD is suspected, and hepcidin-25 is within the reference range, presence of hepcidin-20 could be used as a marker for ACD. However, much larger studies are required to fully evaluate this hypothesis, but it does suggest a possible role for hepcidin-20 measurement in clinical practice.

Isolated hepcidin-25 measurement has the advantage that it would indicate the ability for iron to be absorbed. For example, if hepcidin-25 was found to be significantly raised, oral iron may not be effectively absorbed, but treatment with IV iron could be administered immediately, preventing the adverse effects of oral iron (e.g. gastrointestinal upset), and reducing delays in treatment. Certainly, the usefulness of measuring hepcidin-25 to personalise the optimal route of iron (i.e. oral or IV) has been indicated in patients with IDA (Bregman *et al.,* 2013). Furthermore, a significantly elevated hepcidin-25 concentration in an individual with IDA is almost diagnostic of Iron Refractory Iron Deficient Anaemia, which is caused by a mutation in the *TMPRSS6* gene, although confirmation by gene sequencing is recommended (Girelli *et al*., 2016).

Diagnosing ACD with concomitant ID is difficult. For example, inflammatory bowel disease is associated with frequent blood loss and iron-deficiency. Detection of ID is difficult using currently available markers of iron-status, such as ferritin, in part, because ferritin, is an acute-phase protein, and is elevated during infection/inflammation, even when iron deficiency is present. An algorithm including the soluble transferrin receptor is available (Goodnough *et al.,* 2010), however, measurement of the soluble transferrin receptor is not widely available, and reference ranges are method dependent. In such complicated situations, measurement of hepcidin-25 has shown to be useful in differentiating between ACD, IDA and those individuals with both ACD and ID. Individuals with ACD and concomitant ID have a hepcidin-25 concentration that is lower than those with 'pure' ACD, but raised compared to those with IDA. This approach has been used successfully in patients with rheumatoid arthritis (van Santen *et al.,* 2011). Unfortunately, samples were not available to investigate this in the work undertaken here.

5.4.4 Hepcidin in Sickle Cell Anaemia and Congenital Dyserythropoietic Anaemia

The samples analysed here were from individuals who were not known to be undergoing a crisis, and the hepcidin-25 concentration was significantly lower than those measured in controls. This would be expected as almost all (92 %) samples had a low haemoglobin, and therefore hepcidin-25 synthesis would be suppressed to ensure maximal iron absorption for effective erythropoiesis. Furthermore, concentrations of the soluble transferrin receptor were raised as compared to the manufacturer's suggested reference range (8.7–28.1 nmol/L), which is consistent with increased erythropoiesis. This increased erythropoiesis would further drive the

suppression of hepcidin-25 synthesis. Although only a small number of samples were analysed, the data generated does confirm findings from other investigators that in individuals with SCA, hepcidin-25 production is suppressed in line with increased erythropoiesis (Kroot *et al.,* 2009b; El Beshlawy *et al.,* 2012; Karafin *et al.,* 2015). As in individuals with IDA or ID, there were no *N*-truncated isoforms of hepcidin-25 present, which would be expected if they are degradation products of hepcidin-25. The clinical usefulness of hepcidin-25 measurement in individuals specifically with SCA is currently limited. However, hepcidin-25 measurement has been suggested as a diagnostic marker of severe iron overload in patients with β-thalassemia major, although larger studies are required to confirm this finding (Kaddah *et al*., 2017).

In the four CDA II samples analysed here, hepcidin-25 was below the LLoQ in all except 1 sample where it was 3 µg/L. These findings are in-keeping with other published results, in that hepcidin-25 is inappropriately low given the degree of iron overload, and the ineffective erythropoiesis down regulates hepcidin-25 expression in these individuals (Casanovas *et al.,* 2011). Further studies are required to identify, what, if any role measurement of hepcidin-25 has in management of patients with CDA II.

5.4.5 Hepcidin in HFE-related iron overload

Given the samples analysed were from individuals known to have mutations in the HFE gene, and who were being treated for iron overload by regular venesection, therefore it is not surprising that many had non-detectable or low serum concentrations of hepcidin-25. Individuals that were C282Y homozygous had hepcidin-25 concentrations which were inappropriately low for the iron load present, as indicated by the low ferritin:hepcidin-25 ratio, and support findings reported by other investigators (van Dijk *et al.,* 2008; Piperno *et al.,* 2007). Hepcidin-25 concentrations measured in C282Y homozygotes were broad, with some samples having non-detectable concentrations of hepcidin-25, whereas others had concentrations of hepcidin-25 that were within the range measured in controls. This most likely reflects the varying periods of time these individuals have been undergoing venesection. For example, it has been shown by other investigators that in untreated C282Y homozygotes, hepcidin-25 declines over time from when they are first diagnosed (i.e. untreated) to when undergoing regular (i.e. maintenance) venesection (van Dijk *et al.,* 2008), most likely due to suppression of hepcidin-25 synthesis in response to decreased ferritin concentrations and increased erythropoiesis, caused by regular venesection.

The hepcidin-25 concentration in C282Y heterozygotes was not found to be significantly different from the control group, but was significantly raised as compared to individuals with the C282Y/C282Y genotype. The H63D polymorphism (either heterozygote or homozygote) is generally considered to have little clinical significance (Kelley *et al.,* 2014). This may explain why there was no significant difference in concentrations of hepcidin-25 between C282Y heterozygotes, and C282Y/H63D compound heterozygotes (i.e. they have the same functional phenotype). Given the H63D mutation has no effect on HFE protein function, and has little clinical significance with regards to iron overload, the significantly raised concentrations of hepcidin-25 in samples from H63D heterozygotes, and H63D homozygotes compared to controls and the other HFE mutations studied, reflects increased hepcidin-25 synthesis in response to iron overload, which is likely due to other reasons and not the H63D mutation. Although specific clinical details are not available for the individuals studied here, all are known to have diabetes, dysmetabolic iron overload syndrome or other co-morbidities that could account for the iron overload present.

Overall, the concentrations of hepcidin-25 measured in individuals with various HFE genotypes are as would be expected. It should be remembered that all samples were from individuals undergoing regular venesection to deplete iron stores or for maintenance purposes. Therefore, comparison could not be made between concentrations of hepcidin-25 in recently diagnosed individuals and those undergoing venesection for maintenance purposes. The usefulness of hepcidin-25 measurement in individuals with HFE-related haemochromatosis has not been ascertained, but is likely to be limited. For example, ferritin, TIBC, and TSAT are all available in most general hospitals, and are well established markers of iron status and iron overload. Furthermore, genetic sequencing of the HFE gene is available and would provide a 100 % confirmation of the presence of a HFE mutation. Hepcidin-25 has been suggested as being a useful tool for monitoring the effectiveness of treatment with venesection, to ensure that hepcidin-25 synthesis is not fully suppressed, which would exacerbate iron absorption and overload (van Dijk *et al.,* 2008). However, larger clinical studies are required to fully investigate this proposal.

5.4.6 Hepcidin in non-HFE related iron overload, and non-alcoholic fatty liver disease

The samples analysed here were from individuals with non-HFE related iron overload that had been previously screened and found not to have any genetic mutations in the *HFE*, *TfR2*, *HJV*,

or *SLC40A1* genes that are known to cause haemochromatosis, and had no evident reason for iron overload. In these samples hepcidin-25 was raised in response to increased iron stores, and therefore iron absorption should be suppressed, but this was not the case. These individuals have a phenotype like that of 'ferroportin disease', whereby hepcidin-25 is raised but there is 'hepcidin resistance' (Mayr *et al.,* 2010), yet no known mutation in the FP-1 gene were found. This would suggest that there may be dysregulation between hepcidin-25 and FP-1, or that there is an unidentified mutation in FP-1 within these individuals. It has been reported that hepcidin-25 mediated ferroportin internalisation requires activation of the Janus Kinase2 protein for phosphorylation of FP-1 tyrosine residues 302 and 303 (De Domenico *et al*., 2009). However, another study has found this not to be the case and instead hepcidin-25 mediated ubiquitination of ferroportin involving lysine amino acids present between residues 229 and 269 in the third cytoplasmic loop of ferroportin are required (Ross *et al*., 2012; Qiao *et al*., 2012). Currently, work is on-going to identify the cause of iron overload present in these individuals, and full genetic sequencing of the ferroportin gene and other iron-related genes is it be undertaken. In the samples analysed from individuals with NAFLD, hepcidin-25 was significantly (although only marginally) raised as compared to controls, which supports findings from other investigators (Boga *et al.,* 2015; Senates *et al.,* 2011).

5.4.7 Hepcidin in sepsis

Hepcidin-25 synthesis is known to be induced by cytokines, in particular IL-6, which are raised in the acute phase response (Damas *et al.,* 1992; Nemeth *et al.,* 2004). Therefore, it is not surprising that in these samples, hepcidin-25 was significantly raised as compared to controls, although there were still some samples where hepcidin-25 was within the range measured in controls. Almost all individuals had a low haemoglobin, and iron indices (e.g. ferritin, TIBC) that are consistent with ACD, although it is not possible to identify whether these individuals had concomitant iron deficiency. It is interesting that hepcidin-20, -22, and -24 were detected in many samples, and besides hepcidin-25, hepcidin-24 was the most abundant isoform of hepcidin in the samples analysed.

5.4.8 Relationship of hepcidin isoforms

One possible theory on the formation of each *N*-truncated isoform of hepcidin-25 is that each is sequentially degraded to a smaller form. If this were the case then there should be a good

correlation between each isoform. In part, this is reflected in the data here, especially for hepcidin-25 and hepcidin-24 for which there was a strong and significant correlation in all disease states investigated, and when results from all samples were combined. There was also a strong correlation of hepcidin-24 to hepcidin-22, but a weaker correlation of hepcidin-22 to hepcidin-20. There were generally poor correlations between non-adjacent hepcidin isoforms (i.e. between hepcidin-25 and hepcidin-20, hepcidin-24 and hepcidin-20). These data would suggest that each hepcidin isoform is sequentially degraded to each other. In no sample was hepcidin-19, 21, or -23 identified by retrospective data interrogation; of course, without a reference compound to confirm whether these isoforms are extracted or retained on the LC column this can only be tentatively concluded.

There was little difference in the ratio of hepcidin isoforms between the conditions investigated. This may be because of relatively low sample numbers or that the conditions investigated are all similar to each other in terms of CRP concentrations – a factor known to influence hepcidin-25 concentrations through up-regulation of synthesis. Certainly, sepsis is an extreme version of ACD and these two groups had raised CRP concentrations. The ratio of hepcidin-25 to hepcidin-20, and hepcidin-24 to hepcidin-20, however, were significantly lower in samples taken pre-haemodialysis compared to other groups; this is reflective of the accumulation of hepcidin-20 in patients with little to no kidney function as discussed in Chapter 4.

5.5 Conclusions

The LC-HR-MS method developed here has shown to be capable of measuring hepcidin isoform concentrations in samples from a wide range of iron disorders. Hepcidin-20, -22, and -24 were only present when hepcidin-25 was significantly raised, and hepcidin-25 was strongly correlated to hepcidin-24. The clinical importance of this finding requires further investigation, but measurement of hepcidin-25 may have a role in the differential diagnosis of IDA from ACD, and in assessing treatment with either oral or IV iron. The role of measuring hepcidin-25 in conditions of iron overload (e.g. hereditary haemochromatosis) is not clear, and requires further studies.

Chapter 6 General Discussion

6.1 Introduction

The discovery of hepcidin-25 in 2001 has considerably advanced our understanding of iron metabolism, and the mechanisms involved in some disorders of iron deficiency or excess (e.g. IDA, haemochromatosis). Furthermore, novel therapies are being developed for the treatment of iron-overload and iron deficiency such as hepcidin-25 agonists, antagonists, and 'mini-hepcidins' that mimic the actions of endogenous hepcidin-25. It has been, and still is, hoped that hepcidin-25 may be an additional tool in assessing and treating iron status in a clinical setting, and may even replace traditional markers of iron status (e.g. ferritin, TIBC, TSAT).

6.2 Analytical aspects of hepcidin measurement

Despite hepcidin-25 having been first identified in 2001, there is no 'gold-standard' assay available, and there is a lack of continuity between assays, which hampers the use of hepcidin-25 measurement in a clinical setting (Kroot *et al*., 2009; Kroot *et al*., 2012; van der Vorm *et al*., 2016). One area of confusion is over the term 'hepcidin'. This term is used to refer to hepcidin-25 specifically, or used to refer to all known and commercially available isoforms of hepcidin together, i.e. hepcidin-20, -22, -24 and -25. It may also be used to include other potential, non-commercially available isoforms of hepcidin-25; hepcidin-19, -21, and -23. This means, especially for immunoassays where cross-reactivity between these isoforms may exist, it is not clear exactly what is being measured. Additionally, despite hepcidin-20, -22, and -24 having been identified, these additional hepcidin-25 isoforms are rarely measured and, until the work undertaken here, no full validated method for the quantitation of all commercially available hepcidin isoforms has been published (Handley *et al*., 2017, Appendix B).

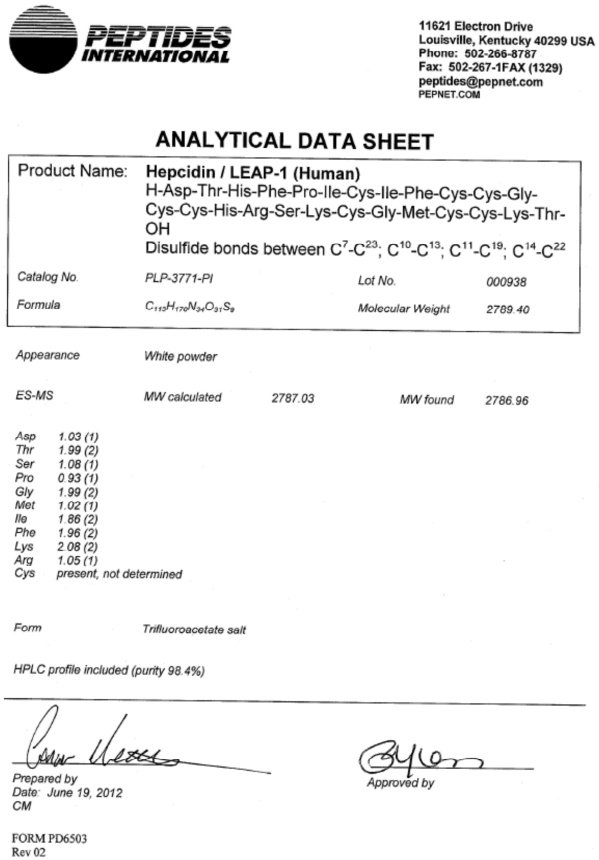
6.2.1 Reference compounds

To date, there is still no fully traceable certified standard available for hepcidin-25. Availability of a certified reference compound enables full traceability of the specified concentration. During the work herein, hepcidin-25 used for the initial work showed on the analytical data sheet an HPLC purity of 98.4 %. However, some months later, a revised analytical data sheet was published adding an additional statement that there was a peptide content of 68.8 % (Figure 6-1. In essence, the HPLC purity defines that 1.6 % of the compound is comprised of impurities, but of the 98.4 % that is 'pure' there is only 68.8 % that is hepcidin-25 itself. As such, when

producing calibration solutions from this solid, only 67.7 % of that which is weighed is actually hepcidin-25 (68.8 % of the 98.4 %); any investigators that made calibrations solutions or validated prior to this revised data-sheet, or that did not acknowledge the relevance of the peptide content could be inaccurate by 30 %.

Indeed, this realisation was made by investigators in a round robin for hepcidin-25, whereby hepcidin-25 purchased from Bachem (Torrance, USA) was assumed to be 100 % pure, but the net peptide content was actually 68.8 % (Kroot *et al*., 2012). These findings raise concerns as to the accuracy of hepcidin-25 concentrations reported when analysed by early assays.

(A)



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ANALYTICAL DATA SHEET

Product Name:	**Hepcidin / LEAP-1 (Human)** H-Asp-Thr-His-Phe-Pro-Ile-Cys-Ile-Phe-Cys-Cys-Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr-OH Disulfide bonds between C^{7}-C^{23}; C^{10}-C^{13}; C^{11}-C^{19}; C^{14}-C^{22}
Catalog No.	PLP-3771-PI
Lot No.	000938
Formula	$C_{113}H_{170}N_{34}O_{31}S_9$
Molecular Weight	2789.40

Appearance	White powder			
ES-MS	MW calculated	2787.03	MW found	2786.96

Asp	1.03 (1)
Thr	1.99 (2)
Ser	1.08 (1)
Pro	0.93 (1)
Gly	1.99 (2)
Met	1.02 (1)
Ile	1.86 (2)
Phe	1.96 (2)
Lys	2.08 (2)
Arg	1.05 (1)
Cys	present, not determined

Form: Trifluoroacetate salt

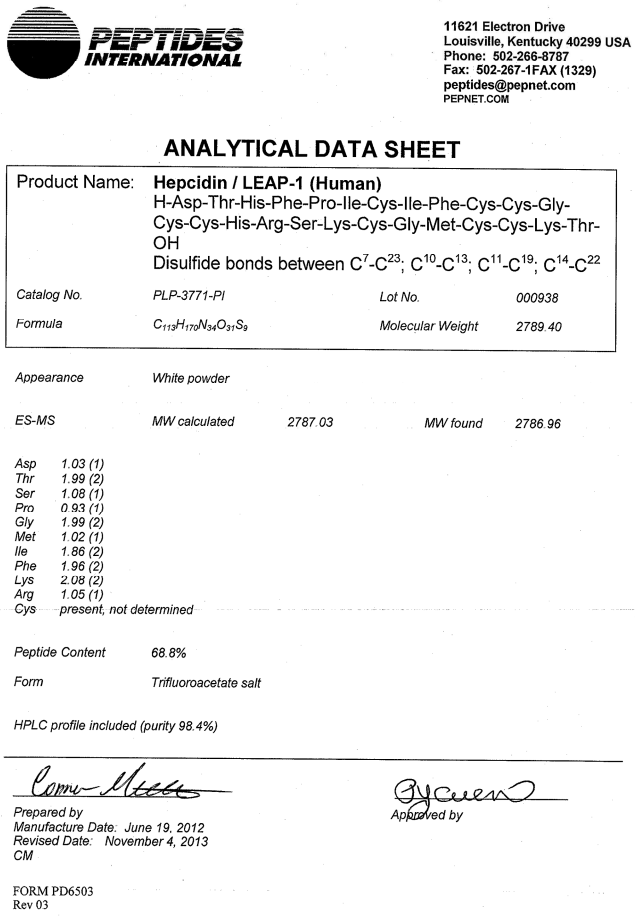
HPLC profile included (purity 98.4%)

Prepared by
Date: June 19, 2012
CM

Approved by

FORM PD6503
Rev 02

(B)



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ANALYTICAL DATA SHEET

Product Name:	**Hepcidin / LEAP-1 (Human)** H-Asp-Thr-His-Phe-Pro-Ile-Cys-Ile-Phe-Cys-Cys-Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr-OH Disulfide bonds between C^{7}-C^{23}; C^{10}-C^{13}; C^{11}-C^{19}; C^{14}-C^{22}
Catalog No.	PLP-3771-PI
Lot No.	000938
Formula	$C_{113}H_{170}N_{34}O_{31}S_9$
Molecular Weight	2789.40

Appearance	White powder			
ES-MS	MW calculated	2787.03	MW found	2786.96

Asp	1.03 (1)
Thr	1.99 (2)
Ser	1.08 (1)
Pro	0.93 (1)
Gly	1.99 (2)
Met	1.02 (1)
Ile	1.86 (2)
Phe	1.96 (2)
Lys	2.08 (2)
Arg	1.05 (1)
Cys	present, not determined

Peptide Content: 68.8%

Form: Trifluoroacetate salt

HPLC profile included (purity 98.4%)

Prepared by
Manufacture Date: June 19, 2012
Revised Date: November 4, 2013
CM

Approved by

FORM PD6503
Rev 03

Figure 6-1 Analytical data sheets for hepcidin-25 of the same lot number, both with purity indicated but without (A) and, with (B) peptide content given.

6.2.2 Methodology

Published assays for hepcidin-25 measurement broadly fall into 2 main groups: immunoassay and mass-spectrometry based. Advantages of immunoassays for hepcidin-25 are that minimal specialised equipment is required, kits are commercially available, relatively high-throughput is obtainable, and the assay has already been validated by the manufacturer (although in-house validation of the assay is always recommended). The main disadvantage however, is that isoforms of hepcidin-25 may cross react with the 'specific' hepcidin-25 antibodies falsely elevating the 'measured' hepcidin-25 concentration. Certainly, the comparison of the LC-HR-MS assay developed here to a commercially available ELISA showed concentrations of hepcidin-25 were some 10-fold higher measured using the ELISA. It is interesting to note that the N-truncated hepcidin isoforms were not detected in any of these comparison samples, therefore cross-reactivity with these isoforms was not the cause of the discrepancy, and it may be attributed to other endogenous compounds present in the sample or an issue with calibration.

Mass spectrometry has the potential to be more selective than immunoassays for hepcidin-25 measurement, and has the advantage that each hepcidin-25 isoform can be separately quantified. However, this does not mean that MS based assays are more accurate than immunoassays, as was certainly shown in round robins for hepcidin-25 (Kroot *et al*., 2009; Kroot *et al*., 2012; van der Vorm *et al*., 2016). Additional complications can arise from an inappropriate selection of internal standard; some published MS based methods used hepcidin-24 as an internal standard (Swinkels *et al*., 2008; Tessitore *et al*., 2010; van der Weerd *et al*., 2012; Addo *et al*., 2016), until it was recently identified as being present in-vivo. The introduction of a commercially available isotopically labelled hepcidin-25, however, will do much to improve MS based assays (Ward *et al*., 2008).

Mass spectrometry assays for hepcidin-25 can be broadly divided into SELDI/MALDI based methods, and LC-MS(/MS) based methods. SELDI/MALDI-TOF-MS based methods were some of the first assays published for hepcidin-25 (Tomosugi *et al*., 2006), likely due to these being 'soft' ionisation techniques enabling identification of a compound from its molecular ion, and analysis is rapid as there is no chromatography. However, because sample pre-treatment can be limiting, and there is no further separation of analyte from potential interfering compounds (such as liquid chromatography) accurate quantitation may be compromised, although the limitations can be reduced by thorough method validation and use of an appropriate internal standard. Some thoroughly validated methods have been published that

have shown these techniques to be applicable for the measurement of hepcidin-25 (Ward *et al*., 2008; Kroot *et al*., 2010).

SELDI/MALDI instruments are primarily based in academic and research laboratories, whereas in clinical laboratories LC-MS/MS instruments are common place, and it is these instruments that would be used for the quantitation of hepcidin-25 should the assay become used routinely within a clinical setting. Certainly, LC-MS/MS based methods for hepcidin-25 are becoming widely published (Wolff *et al*., 2013; Delaby *et al*., 2014; Lefebvre *et al*., 2015). The advantage of LC-MS/MS is that through sample preparation can be undertaken prior to analysis that, together with LC, helps to reduce the effect of interfering compounds on accurate quantitation. A consideration with this technique, however, is that when ESI is used, hepcidin-25 (and N-truncated isoforms) has several charge states (primarily $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$), and in all published LC-MS/MS methodologies only a single charge state is monitored that is not consistent between methods; there may be sample-to-sample variability in isotope and charge state distribution, which would not be corrected for should only a single charge state be monitored. In the work undertaken here it was found that, although the charge state distribution did not vary significantly between samples it was very different in extracted samples to those observed when each individual hepcidin was infused separately into the MS (Figure 2-1 and Figure 2-2). This has implications in LC-MS/MS methodology as there is potential for an inappropriate charge state to be chosen during method development. When the method developed here was compared to an LC-MS/MS based assay that detected only a single charge state, there was a good correlation between results. Be this as it may, monitoring a minimum of 2 charge states per analyte would minimise the effect of this potential variable.

An advantage of the method developed here is that by using LC-HR-MS, and acquiring data in full scan mode, all charge states and isotopes can be selected for post-acquisition data processing. Furthermore, data can be interrogated post-acquisition for analytes not initially targeted, as was undertaken here when investigating analyte stability and whether hepcidin-19, -21, or -23 were also present.

6.2.3 Sample preparation

LC-MS(/MS) methods published for hepcidin-25 primarily use solid phase extraction, although a few methods using protein precipitation have been reported. In the work undertaken here, protein precipitation was found to be an unsuitable sample preparation technique due to poor

process efficiency and inadequate sensitivity. Immunocapture was likewise not successful, most likely due to endogenous compounds found in serum interfering with antibody binding. Instead, after extensive development, solid phase extraction was considered the most suitable sample preparation technique. Certainly, the use of solid phase extraction is widely reported in the literature for hepcidin-25 measurement (Li *et al*., 2009; Wolff *et al*., 2013; Lefbevere *et al*., 2015). Advantages of this technique, and the method developed here, are that: (i) 96-well plates are used to aid throughput, and (ii) the supernatant does not require evaporation and re-constitution, either to improve sensitivity or to make it appropriate for injection into the LC system. The whole sample preparation time for 96 samples (including calibrators/IQCs) was approximately 2 to 2.5 hours, prior to analysis by LC-HR-MS - therefore quite applicable to batch analysis in a clinical setting.

6.2.4 Analyte stability and hepcidin isoforms

In clinical samples, all analytes were relatively stable for a few days when stored at 2–8 °C, but all had limited stability when stored at ambient room temperature. Therefore, in a clinical setting samples should either be frozen immediately if analysis is to be delayed, or stored at 2–8 °C should analysis be undertaken in the proceeding days after collection. If measurement of hepcidin isoforms is requested as an additional test, i.e. after all tests have been undertaken on a sample, this should be possible if the sample is refrigerated or frozen.

It is interesting that when each individual hepcidin was added to stripped human serum, a decline in the concentration of an individual hepcidin (e.g. hepcidin-25) was not matched by a corresponding increase in another (e.g. hepcidin-24); except for hepcidin-22, where a decline in concentration was paralleled by an increase in hepcicin-20. A similar picture was present in clinical samples. Yet, all isoforms of hepcidin have been measured in patient samples, particularly those samples from individuals with ACD or sepsis, or where hepcidin-25 is raised. These findings are of interest as it not only strongly suggests that N-truncated isoforms of hepcidin-25 are not an artefact formed only upon storage or processing of samples, but that they do exist in-vivo. Since there was no concurrent increase in alternative isoforms, the decline in hepcidin concentrations measured during storage may be due to aggregation, or adsorption to the container wall or other endogenous compounds.

It was interesting that all hepcidins were more stable in stripped human serum than in clinical samples; a possible explanation is that the process of ‘stripping’ human serum with

charcoal may reduce enzyme activity as has been shown with bovine serum (Cao *et al*., 2009). Therefore, both enzyme activity and adsorption may both contribute to the decline in hepcidin concentrations during storage. This is further supported by a report where addition of protease inhibitors to the plasma from individuals in intensive care (e.g. individuals with raised hepcidin-25 concentrations) minimised the decline in hepcidin-25, but did not completely inhibit it (Laarakkers *et al*., 2013). In-vivo, hepcidin-20, -22, and -24 are most likely formed by enzymatic cleavage of hepcidin-25, although it is not clear which enzymes are involved, nor whether each hepcidin isoform is sequentially degraded. Unfortunately, there has been little research into the enzymes responsible for the degradation of hepcidin isoforms. Dipeptidylpeptidase-4 has been suggested as being responsible for degradation of hepcidin-22 to hepcidin-20 as proline is present at the cleavage site of hepcidin-22, whereas the difference between hepcidin -25 and -24 is the loss of an aspartic acid therefore an aspartic acid protease is most likely involved.

6.3 Application of hepcidin measurement

To date there is no firm evidence to support the clinical use for hepcidin-25 measurement in the diagnosis and management of disorders of iron metabolism. However, this is in-part due to the variability between currently available assays, and a lack of established reference range for various disorders (e.g. ID, ACD, SCA).

6.3.1 Clinical usefulness of hepcidin-25 measurement

Many clinically relevant conditions are known to influence hepcidin-25 (e.g. alcohol abuse, anaemia, severely reduced kidney function), therefore hepcidin-25 concentrations should be interpreted in conjunction with other established markers of iron status (e.g. ferritin, TIBC, TSAT), and the clinical details available. Ferritin is a well-known and used marker of iron stores, it is relatively inexpensive and widely available within hospital laboratories, even though it has several limitations (e.g. an acute phase reactant). Given hepcidin-25 is generally well correlated with ferritin, hepcidin-25 would be unlikely to replace ferritin as a marker of iron stores, nor would it replace TIBC/TSAT as a marker of circulating iron availability. It does, however, provide an indication of potential iron absorption from the gut, and iron release from macrophages, something which no currently available marker of iron status can. Ferroportin controls iron absorption and release, however since it is present in the cell membranes, rather than

circulating in plasma, direct measurement of this protein is not practical, although ferroportin activity can be quantified (Ward & Kaplan, 2012). Hepcidin-25 concentrations can be used as an indirect marker of ferroportin activity in a way that no other marker can.

Hepcidin-25 measurement has been shown to be valuable in the differential diagnosis of IDA from ACD, and may also prove useful in identifying ACD with concomitant ID (Bergamaschi *et al*., 2013; Lasocki *et al*., 2010; van Santen *et al*., 2011; Shu *et al*., 2014). Certainly, while undertaking the work here, several requests were received from clinicians requesting hepcidin-25 measurement in samples from individuals that have had long term IDA that has not responded to iron therapy in order to explain this clinical situation. Hepcidin-25 was below 1 µg/L in all cases indicating that the individuals should be able to appropriately absorb iron. In these cases, measurement of hepcidin-25 was not able to directly identify the cause of anaemia, but it was able to rule out an inappropriately raised hepcidin-25 as a factor in the IDA present. Monitoring hepcidin-25 in hereditary haemochromatosis to avoid complete suppression of its synthesis (via excessive phlebotomy), and iron hyperabsorption has been suggested (Girelli *et al*., 2016). However, whilst preliminary work suggests a value of hepcidin-25 in these situations, larger studies are required to assess the clinical effectiveness of this.

6.3.2 Clinical usefulness of hepcidin-20, -22, and -24 measurement

Very few studies have reported concentrations of hepcidin-20, and -22 in both healthy individual's and those with disorders of iron metabolism, and besides the work published here (Handley *et al*., 2017) no studies have reported concentrations of hepcidin-24. In part, this is because (i) assays developed largely only include hepcidin-25, and (ii) as N-truncated isoforms of hepcidin-25 have shown to have little or no activity at the FP-1 receptor (Laarakkers *et al*., 2013) they are considered of no benefit to be measured. However, hepcidin-20, -22 may have some antimicrobial activity (Ho *et al*., 2013), and measurement of hepcidin-20, rather than hepcidin-25, has been suggested to be helpful in the diagnosis of acute myocardial infarction (Suzuki *et al*., 2009).

In the work undertaken here, hepcidin-20 would seem to have the potential to be a marker of kidney function as well as for ACD, although much larger studies are required to ascertain the clinical relevance of this finding. Even though hepcidin-20, -22, and -24 have little activity at the ferroportin receptor, given the considerable contribution of hepcidin-20, -22, and -24 to the 'total' hepcidin concentration measured in patients with ACD, sepsis, and CKD in the

work here and in reports by another investigator (Campostrini *et al*, 2012), they should not be dismissed as 'degradation products' of no clinical use. It has been hypothesised that hepcidin-20 is not simply a non-regulated degradation product of hepcidin-25, but instead it is degraded by yet unidentified enzymes in response to iron-deficiency or iron-overload; in a healthy population the ratio of hepcidin-25:hepcidin-20 increased with increasing ferritin concentrations (Campostrini *et al*., 2012). This is an interesting hypothesis, which in-part maybe supported by the finding here that in conditions where hepcidin-25 is raised (ACD, sepsis, CKD), generally hepcidin-20 is present, and so are hepcidin-22, and -24. Although, without a clear mechanistic explanation for the formation of hepcidin-20, -22, or -24, these may simply be formed via non-regulated degradation.

6.4 Further work

Future work on hepcidin isoforms should first concentrate on the harmonisation of assays. Although a few round robins have been distributed, a formal scheme should be initiated to provide ongoing confidence into the comparability of published ranges from different methodologies. Limitations to this are that there is no traceable reference compound for hepcidin isoforms, and a potential lack of sample stability when samples are sent worldwide. However, the benefit of such a scheme is that comparison between assays, both between methodologies (i.e. immunoassay vs MS), and within methods (i.e. MS) can be undertaken. Such a scheme should include all isoforms of hepcidin currently available (hepcidin-20, -22, -24, and -25) since, even if they cannot be separately quantified by some methods, the effect of their presence on assay accuracy can be ascertained. Any newly developed assays should, where possible, include all available isoforms of hepcidin.

Furthermore, larger studies should be undertaken in individuals with CKD, IDA and ACD to assess the concentrations of all hepcidins (including hepcidin-20, -22, -24, and -25) present, and whether measurement of hepcidin-20 is useful in determining kidney function and the diagnosis of ACD, as indicated in the preliminary work undertaken here. Again, further work on the formation of hepcidin-20, -22, and -24 would be of value, for example trying to identify the mechanism by which they are formed *in-vivo*, and the enzymes involved (if any).

6.5 Conclusions

The measurement of hepcidin-25, and possibly other N-truncated isoforms are promising tools for the diagnosis and management of disorders of iron metabolism. Especially as hepcidin-25 has been shown to be able to differentiate IDA from ACD, identify ID in ACD, and it can provide an indication as to an individual's response to oral iron. The clinical role of hepcidin-20, -22, and -24 has not been ascertained, although the work here does suggest that hepcidin-20 maybe of use in determining kidney function and in the diagnosis of ACD, although larger trials are required to fully investigate this. Be this as it may, all hepcidin isoforms should always be measured where possible, and for these analytes to be used in routine clinical practice; (i) harmonisation of assays needs to be undertaken, (ii) the importance of hepcidin-20, -22, and -24 needs to be ascertained, and (iii) well clinical decision limits identified.

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Appendix A. Information sheet and consent form

viapath

Centre Number	00011
Number	REFRANGE
Participant Identification Number	

INFORMATION SHEET

Title of Project: **Collection of blood for re-establishing reference ranges for immunology, routine coagulation, trace element and iron homeostasis testing.**

Project Lead: Aimee Rhodes

Date: 20/04/2016

Information provided on this form and the results of these tests will be held anonymously and in strict confidence and will not be revealed to anyone.

At least 50 volunteers are needed as a representative sample of the local population. We need blood donations from the entire adult age range, from both sexes and all ethnic origins to establish local reference ranges. Therefore we would like to take about 20ml of blood (a sodium citrate sample, two serum samples and a trace element EDTA sample) to perform this study. Your donated blood will be used for the determination of reference ranges for:

- Routine immunology
- Routine coagulation
- Trace element analysis
- C-reactive protein
- Hepcidin-25, ferritin and total iron binding capacity, which are involved in iron homeostasis

And no other purpose.

Hepcidin-25 is considered to be the master regulator of iron homeostasis and controls plasma iron concentrations by binding to and degrading ferroportin. Therefore the measurement of hepcidin-25 may be useful in the diagnosis of haemochromatosis, in the differential diagnosis of iron deficient anaemia from anaemia of chronic disease, and guiding the treatment of patients that have anaemia and chronic kidney disease. Currently reference ranges for 'healthy' individuals are very method dependent, so we would like to set up our own method dependent reference ranges. This in turn would enable the comparison of hepcidin-25 concentration between healthy individuals and different disease states.

Thank you for your co-operation

viapath

Centre Number	00011
Number	REFRANGE
Participant Identification Number	

CONSENT FORM

Title of Project: **Collection of blood for re-establishing reference ranges for immunology, routine coagulation, trace element and iron homeostasis testing.**

Project Lead: Aimee Rhodes

Date: 13/04/2016

Participant, please initial in each box below:

1. I confirm that I have read and understand the information sheet dated 13/04/2016 for the above project. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

2. I agree to take part in the above study. ☐

Please sign below:

Name of participant	Date	Signature
Name of person taking consent	Date	Signature
Project lead	Date	Signature

Thank you for your co-operation

Appendix B. Publication

Handley S, Couchman L, Sharp P, Macdougall I, Moniz C. Measurement of hepcidin isoforms in human serum by liquid chromatography with high resolution mass spectrometry. Bioanalysis. 2017;9(6):541-553.

Background: Hepcidin-25 is the master regulator of iron homeostasis. N-truncated isoforms of hepcidin-25 have been identified (hepcidin-20, -22, -24), although data on the concentrations of these isoforms is sparse.

Methodology: Serum was mixed with aqueous formic acid, and the supernatant loaded onto a 96-well-SPE-plate. Eluted analytes were analysed using LC-HR-MS. Forty-seven paired dipotassium-EDTA human plasma and serum samples were analysed.

Results: The LLoQ was 1 µg/L (all analytes). Accuracy and precision was acceptable. There was a good correlation (R2 > 0.90, all analytes) between matrices. The median (range) serum hepcidin-20, -22, -24, and-25 concentrations measured were 4 (1–40), 8 (2–20), 8 (1–50), and 39 (1–334) µg/L, respectively.

Conclusion: LC-HR-MS is widely applicable to the measurement of hepcidin-25, and truncated isoforms.